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Interactions Between Macrophages of Guinea Pigs and Salmonellae

I. Fate of *Salmonella typhimurium* Within Macrophages of Normal Guinea Pigs[1]

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A suspended cell culture procedure was described for the cultivation of guinea pig macrophages infected with *Salmonella typhimurium*. The fate of the intracellular bacteria was assessed by quantitative recovery of viable bacteria with 0.5% solution of sodium desoxycholate. Two strains of *S. typhimurium* with different degrees of virulence for mice were compared. There was an initial destructoin of intracellular bacteria of both strains; however, the extent of this destruction differed. Approximately 1% of the avirulent bacteria initially phagocytized survived at the end of 4 hr, whereas approximately 8% of the virulent bacteria survived at the end of 3 hr. After this initial killing, the intracellular bacteria began to multiply at a logarithmic rate between 3 and 21 hr after phagocytosis, and then a stationary phase was attained. The rate of this multiplication was comparable for both strains.

Experimental studies on the basis of immunity in salmonellosis have been pursued by many investigators and were recently reviewed by Jenkin and Rowley (7). It is evident that a great deal of controversy exists concerning the relative importance of cellular and humoral components in the acquired resistance of the host to salmonellae. As salmonellae are generally considered to be facultative intracellular parasites (11), it is not unlikely that acquired resistance of the host involves, at least in part, an alteration in the rate of intracellular bacterial multiplication, such as has been demonstrated in the case of tuberculosis (5).

A review of the literature indicated, however, that the fate of salmonellae within normal host macrophages has yet to be clearly defined. Furness (2) reported that there was an initial killing of both avirulent and virulent *Salmonella typhimurium* within normal mouse macrophages and, while a small percentage of the avirulent organisms survived without multiplication, the virulent ones multiplied after 3 hr. Morello and Baker (9) confirmed this initial killing of intracellular *S. typhimurium*. In both studies, the authors used distilled water to disrupt the host cells for the recovery of viable intracellular bacteria. The data presented by Blanden, Mackaness, and Collins (1) also showed an initial bactericidal effect of normal mouse macrophages against virulent *S. typhimurium*, although in their recovery of viable intracellular bacteria, no apparent effort was made to ensure the disintegration of infected cells to release individual intracellular bacteria. On the contrary, Jenkin and Benacerraf (6) showed that, when opsonized with normal mouse plasma, avirulent *S. typhimurium* were killed and virulent ones multiplied within normal mouse macrophages. They assayed the viable intracellular bacteria at various intervals by vigorously rubbing the cover slips, on which the infected cells were attached, on the agar surface for colony count. Gelzer and Suter (3), Mitsuhashi, Sato, and Tanaka (8), and others attempted to show the intracellular multiplication of salmonellae by microscopic examination of stained preparations of infected cell cultures.

These conflicting observations may, in fact, be traced to the varying experimental procedures among different groups of investigators. The common deficiency in these procedures appeared to be the failure to present a precise quantitative evaluation of the fate of intracellular bacteria. The purpose of this study is to design an experi-

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mental procedure which could determine the fate of salmonellae by quantitative recovery of the viable intracellular bacteria, and with this, to determine the fate of avirulent and virulent *S. typhimurium* after their ingestion by macrophages.

MATERIALS AND METHODS

Organism. Two strains of *S. typhimurium* were obtained through the courtesy of L. Joe Berry, Bryn Mawr College, Bryn Mawr, Pa. The avirulent strain (RIA) had an LD_{50} for mice of 10^6. The stock virulent strain (SR-11) had an LD_{50} for mice of approximately 10^4, unless serially passed through mice. Stock cultures of these bacteria were kept on Tryptic Soy Agar slants (Difco) either at 2 C or under mineral oil at room temperature.

To obtain bacterial suspensions in saline, cultures were grown in tubes of Trypticase Soy Broth (Baltimore Biological Laboratory, Baltimore, Md.) placed in a rotating drum at 37 C for 5 to 6 hr. The bacteria were collected by centrifugation at $1,100 \times g$ for 20 min at 5 C. The sediments were then washed twice in 5 ml of saline. The final sediments were suspended in saline and adjusted to an optical density of 200 in a Klett-Summerson photoelectric colorimeter with a no. 66 filter with an approximate spectral range of 600 to 700 mμ. It was previously determined by viable count that saline suspensions of salmonellae at this optical density contained approximately 10^9 bacteria per ml of strain RIA or 2×10^9 bacteria per ml of strain SR-11. These suspensions were used for infecting the macrophages. In some experiments, saline suspensions of the bacterial culture were prepared the previous evening and kept at 2 C. It was determined that overnight refrigeration did not significantly alter the viability of the bacteria.

Guinea pigs. Albino male guinea pigs were purchased from commercial sources and kept on diets of guinea pig pellets and greens. Apparently healthy animals weighing between 400 and 700 g were used in these experiments.

Procedure for suspended culture of macrophages infected with S. typhimurium. Macrophages were induced by intraperitoneal injections of 10 ml of mineral oil (Marcol no. 90, Humble Oil and Refining Co.) into guinea pigs 3 to 5 days prior to harvesting. The animals were exsanguinated by removing the blood from the heart aseptically. The serum was frozen until used. The peritoneal cavity was washed gently 2 to 3 times with a total of 40 to 60 ml of Hanks' solution containing 2% homologous serum and 6 units of heparin (Liquaemin Sodium "10"; Organon Inc., West Orange, N.J.) per ml at *p*H 7.4. The cell suspension was separated from the oil and the *p*H was immediately adjusted to 7.2 with 1.4% isotonic sodium bicarbonate solution. The cells were then collected by centrifugation at $220 \times g$ for 15 min. The sediments were pooled in 5 to 10 ml of the above-mentioned Hanks' washing solution, and the cell population in the suspension was determined in a hemocytometer. Such suspensions usually contained over 70% monocytes.

Infection of the macrophage suspension with the bacteria was achieved by the following protocol. A sample of the macrophage suspension containing 36 to 42×10^6 cells was transferred to a test tube. To this, 1 ml of homologous serum and sufficient amount of the washing solution were added to yield a final volume of 6 ml. Then an appropriate volume of the prepared bacterial suspension was added so that the ratio of cells to bacteria was approximately 1:8 in the case of the avirulent strain (RIA) or 1:16 in the case of the virulent strain (SR-11). This mixed suspension was placed in a drum rotating at 25 rev/min at 37 C for 15 min to permit phagocytosis of the bacteria. After this period, kanamycin, in a final concentration of 25 or 40 μg/ml, was added to the mixture to inactivate the uningested bacteria. The leukocytes in the suspension were collected by centrifugation at $100 \times g$ for 10 min at 5 C or, in the modified protocol, diluted 10-fold in chilled Hanks' solution before centrifugation. The pellet was suspended in tissue culture medium composed of 70% Medium 199 (Microbiological Associates, Inc., Bethesda, Md.), 20% homologous serum, and 10% isotonic 1.4% sodium bicarbonate (CO_2-saturated). Kanamycin, in a concentration of 25 or 40 μg/ml, was added to the medium to suppress the extracellular multiplication of bacteria. The cell suspension was transferred to a 25-ml Erlenmeyer flask coated with silicone (Siliclad, Clay-Adams, Inc., New York, N.Y.) and closed with a rubber stopper. The flask was placed in an Eberbach water bath with horizontal shaking at approximately 80 cycles per min. After each sampling, the flasks were flushed gently with 5% CO_2 in air to maintain the cell suspension in a high CO_2 tension.

Procedure for sampling the infected macrophage culture at intervals. The 0-hour sample was taken from the flask before it was placed in the water bath. During incubation, some cells were "thrown" onto the wall of the flask by the shaking movement of the water bath. These attached cells could easily be released by scraping gently with a platinum wire at sampling intervals. In taking samples, the cell suspension was mixed with a 5-ml pipette and a 0.5-ml amount was removed with a 1-ml pipette.

The sample was treated in the following manner. (i) A 0.2-ml amount was placed in a 15-ml centrifuge tube containing 1.8 ml of Hanks' solution. The cell suspension was mixed and centrifuged at $80 \times g$ for 6 min at 5 C to sediment the leukocytes. (ii) A 0.2-ml amount of the sample was mixed with 0.2 ml of the washing solution containing 0.25% eosin Y (Fisher Scientific Co., Pittsburgh, Pa.) and was left standing at room temperature for at least 5 min. Duplicate samples were taken from this mixture for the determination of total and viable leukocyte populations in a hemocytometer. In all of the experiments reported here, over 90% of the samples of leukocytes from cultures retained their ability to reject eosin throughout the period of observation. (iii) The remaining 0.1-ml sample was used to prepare smears for cytological studies.

The centrifuged portion of the sample was used for the quantitative recovery of cell-associated bacteria and the estimation of the extracellular bacterial popu-

lation. The supernatant fluid (2 ml) was carefully withdrawn and mixed thoroughly with 2 ml of saline. A 0.5-ml amount of this was diluted in 4.5 ml of saline, and 0.5 ml was transferred to a petri dish for bacterial colony count by the pour-plate method. A 0.5% solution of sodium desoxycholate was found to be highly effective in the complete disintegration of leukocytes upon contact, yet had no effect on the viability of *S. typhimurium* even after an exposure of 10 min. One milliliter of 0.5% sodium desoxycholate was added to the cell sediment and pipetted thoroughly to liberate all of the cell-associated bacteria. A 0.5-ml amount of this suspension was then mixed with 4.5 ml of saline, from which serial 10-fold dilutions were made up to 10^{-3} or 10^{-4}. From each dilution, 0.5 ml was placed in a petri dish for bacterial-colony count by the pour-plate method. Tryptic soy agar (Difco) was used in all pour-plate procedures. The plates were incubated at 37 C for at least 30 hr before the bacterial colonies were counted.

By correlating the number of viable bacteria recovered from the cell sediment of a 0.2-ml sample with the total number of leukocytes present in a 0.2-ml sample, the ratio of bacteria to cells was computed for each interval.

RESULTS

Initial destruction of intracellular S. typhimurium. Peritoneal macrophages from a normal guinea pig were divided into two equal portions and permitted to phagocytize either the avirulent or the virulent strain of *S. typhimurium*. The infected macrophages were cultured in suspension as described above. At intervals, the ratio of bacteria to cells was determined for each strain of bacteria. The fate of intracellular bacteria was represented by the growth ratio which was calculated by the number of bacteria per cell recovered at intervals divided by the number of bacteria per cell initially phagocytized. In other words, the growth ratio may be interpreted as the percentage of viable intracellular bacteria recovered at various intervals, and by using this ratio at 0 hr as 100%.

The growth ratio was plotted along the ordinate over a period of 7 hr in which the intracellular fates of the avirulent and the virulent strains of *S. typhimurium* were compared (Fig. 1). Here there was an initial killing of the intracellular bacteria of both strains. However, there was a significant difference in the extent of this initial intracellular destruction. With the virulent strain, approximately 7 to 8% of the organisms initially ingested survived at the end of 3 hr and began to multiply; with the avirulent strain, at the end of 4 hr, approximately 1% of the organisms survived and then multiplied. These data represent an average of four experiments. In each experiment, the initial ratio of phagocytosis

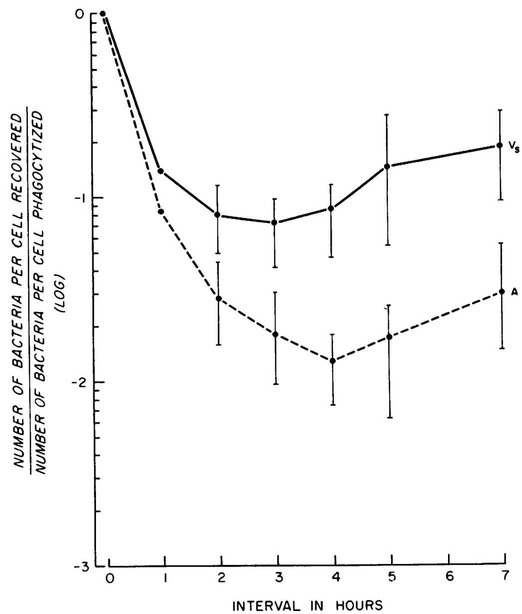

FIG. 1. *Initial destruction of S. typhimurium within macrophages of normal guinea pigs. A = avirulent strain (RIA); V_s = stock virulent strain (SR-11). Cross-bars at each interval represent range of data from which average was taken. Beginning at 2-hr interval, the mean values for V_s were significantly greater ($P > 0.95$) than the mean values for A, as indicated by individual t tests.*

(i.e., the number of bacteria per cell recovered at 0 hr sampling) ranged between 0.7 to 1.0.

Subsequent multiplication of intracellular S. typhimurium. Figure 2 shows essentially the same type of experiments, except that the period of observation was extended to 28 hr. Again, there was a significant difference in the initial killing of the intracellular avirulent and virulent bacteria. The extent of this difference at 3 hr was comparable to that shown in Fig. 1. After this initial period of intracellular destruction, the bacteria of both strains began to multiply between 3 and 21 hr at a logarithmic rate. The rate of this multiplication appeared comparable for both strains, and a stationary phase was attained after 21 hr. These data were drawn from an average of five experiments whose initial ratios of phagocytosis ranged between 0.4 and 0.8.

Supporting experiments. In the above experiments, kanamycin was incorporated into the culture medium to suppress the extracellular bacterial multiplication. Two possibilities must be considered.

(i) It is possible that the initial destruction of intracellular bacteria was a direct bactericidal effect of the antibiotic entering into the host

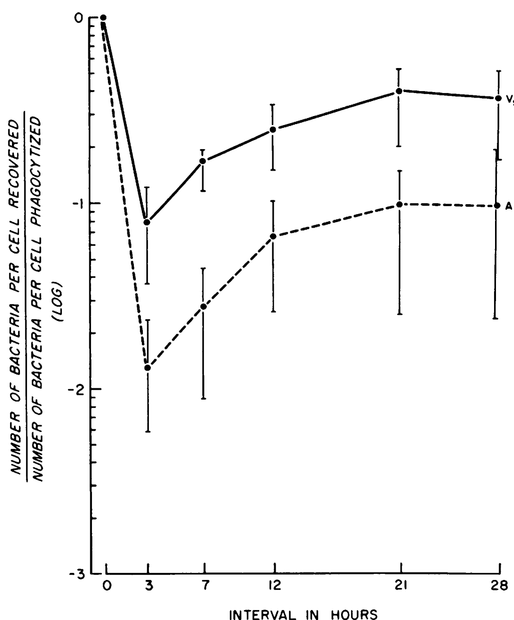

FIG. 2. *Fate of S. typhimurium within macrophages of normal guinea pigs. A = avirulent strain (RIA); V_s = stock virulent strain (SR-11). Cross-bars at each interval represent range of data from which average was taken. Beginning at 3-hr interval, the mean values for V_s were significantly greater ($P > 0.995$) than the mean values for A, as indicated by individual t-tests.*

cells, and that the difference in the extent of the initial killing of these two strains of bacteria might be an inherent difference in the sensitivity of these organisms to the antibiotic. The same type of tissue culture experiment was repeated in the absence of kanamycin. To eliminate a high percentage of the initially uningested bacteria, the infected cells were washed twice in 40 to 60 ml of chilled Hanks' solution before resuspension in tissue culture medium. It was observed that with the virulent strain there was a slight decrease in the bacterial population within 1.5 hr after phagocytosis, after which it began to increase. With the avirulent strain, however, there was a 70 to 80% decrease in the intracellular bacterial population within 1.5 hr after phagocytosis, and it remained at this level up to 3 hr. It is important to note that the extracellular bacterial population remained high, and preliminary experiments also showed that when these bacteria were inoculated into the tissue culture medium, rapid logarithmic multiplication occurred after 1 hr. It may then be concluded that there is a genuine difference in the ability of the macrophages to destroy these two strains of bacteria and that the high level of intracellular bacteria in the kanamycin-free medium reflects a continual phagocytosis of viable extracellular bacteria which have not been exposed to the intracellular environment for sufficient length of time for their destruction.

(ii) Subsequent multiplication of the intracellular bacteria provided the best evidence to support the contention of unlikely interference of kanamycin with the fate of intracellular bacteria. To show that this multiplication was not the result of a development of kanamycin-resistant mutants, colonies were picked from agar plates used in the recovery of cell-associated bacteria of both strains at 7- and 22-hr samplings and plated on agar medium containing kanamycin. It was found that all colonies tested were sensitive to this antibiotic at concentrations as low as 3 μg/ml of medium.

Effectiveness of sodium desoxycholate for the quantitative recovery of intracellular S. typhimurium. Water has been used by some investigators (2, 9) for the quantitative recovery of intracellular bacteria. It can be microscopically ascertained that exposure to water tends to leave most of the leukocytes intact, although not viable, so that not all of the intracellular bacteria are liberated as individual units into the suspension. By contrast, a 0.5% solution of sodium desoxycholate can completely disintegrate leukocytes upon contact, thus releasing all the intracellular bacteria. The following experiments were performed to show that 0.5% sodium desoxycholate could be used to recover far more bacteria than could water or saline from the same amount of sample taken from an infected cell culture.

Peritoneal macrophages of normal guinea pigs were infected with the virulent strain of *S. typhimurium* and cultured in suspension as usual. In the first series of experiments (Fig. 3), samples of the infected cells were taken at intervals of 0, 1, 3, 5, and 7 hr. At each sampling, in addition to the usual procedure of lysing the macrophages with 0.5% sodium desoxycholate, an additional 0.2 ml of infected cells was treated with sterile distilled water. Serial dilutions and pour plates were made in the usual manner to determine the bacterial population by each method. The cellular population in a 0.2-ml sample was also determined as usual. In the second series of similar experiments (Fig. 3), samples of the infected cells were taken at intervals of 0, 3, and 22 hr. At each interval, additional 0.2-ml samples of the infected cells were treated with sterile distilled water or saline for the quantitation of total bacterial population (Fig. 3). Each series of experiments represented the average of duplicate determinations. The number of bacteria per cell, as determined by the

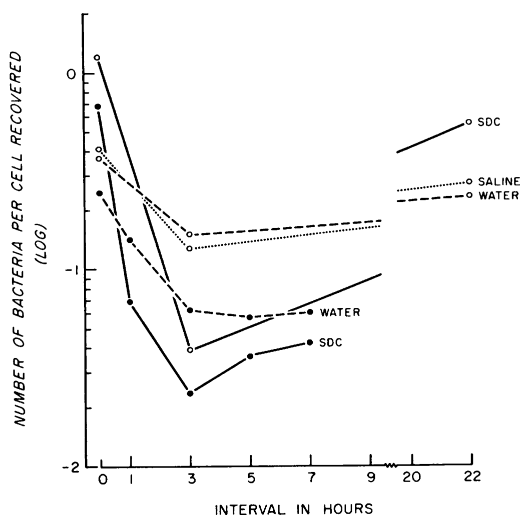

FIG. 3. *Recovery of viable intracellular S. typhimurium (SR-11) with 0.5% solution of sodium desoxycholate (SDC), saline, or water.*

use of sodium desoxycholate, water, or saline, is plotted on the ordinate at each time interval. It is evident that at 0 hr, almost three times as many bacteria were recovered with sodium desoxycholate as with water or saline. However, at the intervals between 1 and 7 hr, more intracellular bacteria were recovered with water or saline than with sodium desoxycholate. A possible explanation for this observation will be presented later. As the intracellular bacteria began to multiply, sodium desoxycholate was again more effective in recovering the intracellular bacteria than was water or saline. This is seen in the sample at 22 hr, in which more than twice as many intracellular bacteria were recovered with the use of sodium desoxycholate as with water or saline.

It may be concluded that a 0.5% solution of sodium desoxycholate has no injurious effect on intact *S. typhimurium* and is therefore more effective in recovering the intact intracellular bacteria, since it can completely disintegrate the macrophages.

DISCUSSION

The suspended cell-culture procedure was used in this series of experiments in contrast to the stationary cell-culture procedure used in other investigations. It offers many advantages. The viability of cells in this culture condition is maintained well above 90% for up to 30 hr of cultivation. Each flask can provide up to seven samples under uniform in vitro conditions. From each sample, both the cellular and bacterial populations are determined to compensate for the continuing decline in the cellular population of the culture. The ratio between bacteria and cells in each sample is used for the determination of the fate of intracellular bacteria. In addition, the morphology of the infected cells may be observed from the smear preparations.

Sodium desoxycholate was used previously for the quantitative recovery of intracellular tubercle bacilli (5), since it could induce an immediate disintegration of leukocytes upon contact, thus releasing the intracellular bacteria. It was also reported to be effective in the recovery of *S. typhimurium* from tissue debris (4). Preliminary tests showed that sodium desoxycholate did not alter the viability of *S. typhimurium* after an exposure of 10 min. On the other hand, water or saline did not disrupt leukocytes, but rather left them intact. At 0 hr, almost three times as many bacteria were recovered from the same quantity of sodium desoxycholate-treated leukocytes as were recovered from water- or saline-treated leukocytes (Fig. 3). However, more bacteria were recovered when water or saline was used at the intervals between 1 and 7 hr. A logical explanation of this observation may be as follows. When the intracellular bacteria are exposed to the digestive enzymes of the host cells, the bacterial cell surface probably is damaged first. As these damaged bacteria are released from the intracellular environment and exposed to sodium desoxycholate, the bacterial cell membranes probably are adversely affected by the sodium desoxycholate in a manner similar to mammalian cell membranes; hence, the injured bacteria will not be recovered in the presence of sodium desoxycholate. By contrast, these damaged bacteria will be well protected by the cytoplasm of the macrophages when the infected cells are exposed either to water or saline, since such treatment does not disrupt the host cells. Thus, when the damage to the cell surface is not too extensive, the bacteria probably will survive and multiply to produce colonies in the agar medium. As the intracellular bacterial population increases as a result of subsequent multiplication of the surviving organisms, the use of sodium desoxycholate is again more effective in the recovery of intracellular bacteria than is water or saline (as shown in the sample at 22 hr, Fig. 3). During the interval from 3 to 7 hr, an increase in the intracellular bacterial population was detected with the use of sodium desoxycholate, whereas a similar increase was not shown with water.

Since the exposure of infected leukocytes to water does not thoroughly disrupt the cells to liberate all of the intracellular bacteria into the

suspension as individual units, the recovery of viable intracellular bacteria with water, as reported by Furness (2) and Morello and Baker (9), is more likely to represent the number of infected cells rather than the total number of individual intracellular organisms in the sample. Similarly, Jenkin and Benacerraf (6) attempted to recover the viable intracellular salmonellae by rubbing cover slips, on which the infected macrophages were attached, on agar plates for colony count. It is questionable that this could actually completely disintegrate the host cells, release the intracellular bacteria, and uniformly redistribute them on the agar surface to yield individual colonies. The release of intracellular bacteria as individual units is particularly necessary in cases where each infected cell contains several viable bacteria as a result of either high initial phagocytosis or subsequent intracellular multiplication. On the other hand, microscopic counting of the stained preparations of infected cells (3, 8) does not differentiate the viable from the dead intracellular bacteria, as both will retain stain. In fact, Olitzki, Godinger, and Gershon (10) demonstrated that the viable intracellular count of *S. typhi* declined faster than the total intracellular count, indicating that morphological integrity did not necessarily represent viability of the bacteria. Thus, this method could at best evaluate the intracellular accumulation of bacteria which could include rephagocytosis of dead extracellular bacteria. One of the objectives of this study, therefore, was to design an experimental scheme, such that the fate of the intracellular bacteria may be followed by the quantitative recovery of the viable intracellular bacteria.

Figure 1 shows that there was an initial destruction of both the avirulent and the virulent strains of *S. typhimurium* within the host macrophages. The virulent strain appeared to be much more resistant to this intracellular digestion and could overcome it after 3 hr of intracellular residence. By contrast, the avirulent strain was less capable of adapting to the intracellular environment and required 4 hr to overcome the intracellular digestion. There is an average of six- to sevenfold difference between the percentages of the avirulent and the virulent bacterial populations capable of establishing themselves intracellularly. After this initial phase of destruction, the surviving intracellular parasites of both strains were capable of multiplication. Figure 2 confirms the difference in the initial killing of the two strains of salmonellae and shows a secondary phase of logarithmic rate of bacterial multiplication between 3 and 21 hr after phagocytosis, to be followed by a stationary phase beginning at 21 hr. The rate of intracellular multiplication, however, was comparable for both strains. Our observation of an initial intracellular destruction of salmonellae is therefore in agreement with the conclusions of several investigators (1, 2, 6, 9).

This difference in the ability of the avirulent and the virulent bacteria to resist initial killing by the phagocytes is consistent with the in vivo observation. To generate a cutaneous lesion in guinea pigs, injections containing approximately 10^8 avirulent or 10^6 stock virulent bacteria were required. The LD_{50} for mice of the avirulent strain is 10^6, whereas that of the stock virulent strain is approximately 10^4. The difference in dosage could be partially explained by the difference in the initial percentage of the inoculated organisms which established themselves within the host. Once sufficient numbers of organisms of either strain had successfully survived initial destruction, they could multiply at comparable rates to produce an observable end point of infection. On this basis, an avirulent strain of *S. typhimurium* may be defined as one which is more susceptible to initial destruction by host phagocytes and thus less capable of establishing itself within the host, rather than one which lacks the ability to multiply within the host.

Another point in the consideration of host-parasite relationships is the susceptibility of the bacteria to phagocytosis. Although no specific experiments were designed in this study to investigate the relative susceptibility of the two strains of bacteria to phagocytosis by guinea pig macrophages, our experience indicated that twice as many virulent as avirulent bacteria were usually needed in mixing with a given number of macrophages under comparable in vitro conditions to achieve an equivalent ratio of phagocytosis. This suggested that the virulent bacteria are not only more resistant to initial intracellular destruction, but are also more resistant to phagocytosis than the avirulent ones. The latter contention is in agreement with the observation of Jenkin and Benacerraf (6).

In tissue culture studies on the interactions between phagocytes and bacteria, some antibiotics are usually incorporated into the culture medium to inhibit the multiplication of extracellular bacteria. Otherwise, observations on the fate of intracellular parasites will be complicated by a continual phagocytosis of extracellular bacteria. The experiments in which the fates of intracellular bacteria of the avirulent and virulent strains were compared in antibiotic-free medium confirmed a difference in the extent of the initial killing. The subsequent multiplication of intra-

cellular kanamycin-sensitive salmonellae further suggested that the antibiotic was unlikely to have interfered with the fate of intracellular bacteria.

In brief, an experimental procedure was presented in this study for the determination of the fate of intracellular salmonellae. This provided a more accurate method of assay to study the question of acquired resistance of the host in salmonellosis at a cellular level.

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