INTERACTION BETWEEN A HUMAN MONOCYTIC CELL LINE AND *SALMONELLA TYPHOSA**

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Enticing opportunities are available to present-day investigators for the use of tissue cells grown in vitro in studies of various phases of host cell-bacterial relationships. For such studies the importance of a suitable cell-bacterial system is paramount; some of the systems described in the literature may be applicable for one type of study but unsuitable for another. For example, in certain studies a high level of natural phagocytic capacity in the test cell would be a requirement. For the study of *Mycobacterium tuberculosis* Suter (1952, 1953) found a monocytic cell line suited for the uptake and the intracellular multiplication of tubercle bacilli. Shepard (1958a, 1955) used HeLa, monkey kidney, and human amnion cells for comparing the growth and multiplication of selected strains of *Mycobacterium*. Holland and Pickett (1956) found that smooth forms of *Brucella abortus* multiplied preferentially within chick embryo cells. Furness and Ferreira (1959) were able to infect rat, mouse, and guinea pig macrophages with *Salmonella* and then to study their capacity to kill virulent and avirulent strains of these bacteria. Crawford and Fischel (1959) infected HeLa and monkey kidney cells with *Bordetella pertussis* and observed intracellular multiplication of the organisms. Stinebring and Kessel (1959) used rat and guinea pig mononuclear phagocytes with *Brucella abortus* in their attempt to obtain continuous growth of the organisms by periodic serial transfer in tissue cell cultures. Smadel (1963) studied the course of infection of mouse fibroblasts with *Salmonella typhosa*. Bacterial infection of tissue cells in culture, therefore, seems to offer the investigator a logical approach to the investigation of the pathogenesis of bacterial diseases of man.

In the present study the J-111 line of monocytic cells was infected with *S. typhosa*. These cells can be maintained in continuous culture and are infected with ease; more than 50% of them contain intracellular bacteria within the first 3 hours of incubation. Moreover, the J-111 line is of human origin and is derived from lymphoid tissue which is the type of tissue involved in human typhoid disease. The aim has been to study the uptake of *S. typhosa* by these monocytic cells, to characterize cytopathic effects, and to determine the ultimate fate of phagocytized organisms both in the presence and in the absence of immune serum. These processes are considered to be important aspects of the pathogenesis of *S. typhosa* infection of man.

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METHODS

The tissue cell line.—A culture of the J-111 line of cells was acquired from Dr. Donald J. Merchant, University of Michigan. This line was started by Osgood and Brooke (1955) from the blood of a patient with acute leukemic monocytic leukemia. The cells were grown in Eagle basal medium (EBM) prepared by dissolving 1 ml each of amino acid, vitamin, and glutamine concentrates (Microbiological Associates, Washington, D. C.) in 100 ml of Hanks balanced salt solution (BSS). Basal medium plus 10% human serum (EBM-H) was used for growth and maintenance of the tissue cell cultures. BSS was employed as diluent in the preparation of all media and solutions and for washing cell cultures. For the actual experiments EBM-D was employed, i.e., basal medium plus 10% dog serum. This serum was found to enhance phagocytosis, in line with the experience of Shepard (1958a). Before use, each lot of human and of dog serum was tested with J-111 cells for toxicity, and nontoxic lots were stored at −20 C until needed. Before freezing, dog serum was further tested for the presence of *S. typhosa* H and O antibodies by the test tube method with formalinized broth cultures and heat-killed bacterial suspensions. Only sera in which these antibodies were not detected were used. Thus, the possibility that the phagocytosis-promoting factor of dog serum was anti-somatic antibody was believed to be ruled out. As in Shepard's procedure the serum was not inactivated by heating. Preliminary tests with inactivated and noninactivated sera had shown no measurable difference in results.

For enumeration cells of J-111 monolayer stock cultures were suspended by treatment with 0.25% solution of trypsin (Difco 1:250) for 2 minutes at room temperature, sedimented at 900 rpm for 5 minutes, and then resuspended in EBM-H. Counts were made in a hemocytometer, as described by Merchant et al (1964).

For the propagation of J-111 cells dilution bottles (200-ml capacity), each containing 10 ml of EBM-H, were seeded with 1 million cells per bottle and incubated at 37 C. The cultures were fed on the 3rd and 5th days by replacing 5 ml of the used medium with fresh EBM-H, and the cells were harvested on the 7th day.

For cultures to be used in a particular experiment approximately 100,000 cells in 1 ml of EBM-H were placed in each Leighton tube on day zero and incubated at 37 C. On the 2nd day of growth the medium was replaced by fresh EBM-H. On the 3rd day, prior to infection, the used medium was replaced with EBM-D.

The bacterial culture.—Strain no. 63 of *S. typhosa*, containing Vi antigen, was acquired from the Michigan Department of Health. It was maintained on Bacto heart infusion agar (Difco Laboratories, Detroit, Michigan). Prior to its use in an experiment a culture was tested for the presence of Vi antigen by the agglutination method.

The bacterial suspension used to inoculate the tissue cells was prepared in 0.85% sodium chloride from 18 to 20 hours growth on agar and standardized against a 10-opacity-units glass standard (National Institutes of Health, Bethesda, Maryland) with a Klett-Summerson colorimeter with a green filter. This suspension was always prepared on the day of use. As estimated by periodic colony counts on agar a suspension of this turbidity contained approximately 1.7×10^9 viable organisms per ml. Each tube in an experiment was inoculated with 0.1-ml volume, i.e., 1.7×10^8 organisms.

Procedure for infection of host cells.—

The J-111 cells were infected by adding *S. typhosa* to 3-day-old monolayer coverslip cultures in EBM-D in Leighton tubes in the dose described above. After 2 or 3 hours of incubation at 37 C in this antibiotic-free system the medium containing extracellular bacilli was removed, and the cell monolayers were washed twice with BSS containing streptomycin (40 μg per ml) and penicillin (40 units per ml). After the last wash EBM-D containing 40 units of penicillin and 40 μg of streptomycin per ml was added to each tube, and all cultures were incubated further. At appropriate intervals after the addition of antibiotics coverslips were removed, washed with BSS, fixed and stained with May-Gruenwald-Giemsa stain by the method described by Merchant et al (1964), and examined as described below. Before the coverslip preparations were removed the tubes were placed on the microscope stage in an inverted position and observed by 100× or 400× magnification for cytopathology of the infected cells or for any other morphological changes.

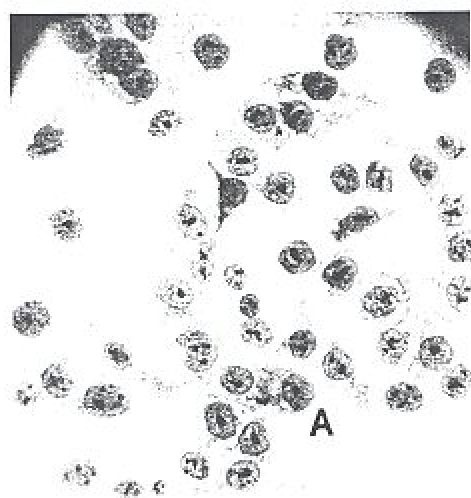
Criteria for infection.—In stained preparations a cell was considered infected if the bacteria were intracellular, i.e., within its cytoplasm. Bacteria were regarded to be intracellular when they were (1) within vacuoles, (2) more faintly stained than bacilli on the surface of cells or on the coverslips, and (3) arranged roughly parallel to each other. All 3 criteria had to be fulfilled to indicate intracellular location.

Scheme for the quantitative expression of intracellular organisms.—To express the results of experiments quantitatively as a basis for comparison and interpretations the method of Kendrick et al (1937) for opsonocytophagic tests in studies of pertussis was used. By experience a method of scanning was selected which would sample the entire preparation. Four different lengths of the coverslip preparation were scanned systematically; all of 200 intact cells were counted and listed in groups according to the range of numbers of intracellular organisms per cell, i.e., 0, 1–5, 6–20, 21–40, and 41–60 (or more). In the development of the method the unit range was 20, thus 1–20 was considered as 1, 21 to 40 as 2, 41 to 60 (or more) as 3. Because a finer division seemed indicated, the range 1–20 was divided into 1–5 and 6–20, i.e., 1/4 and 3/4 of the unit 20. Thus, by reducing 0, 1/4, 3/4, 2 and 3 to a common denominator the numbers of weighting factors became 0, 1, 3, 8 and 12. By adding the products obtained by multiplying the number of cells in the various groups by their respective factors indices were derived which reflected the relative numbers of intracellular bacteria.

RESULTS

Characteristics of the Infection in J-111 Cells with S. typhosa

Observations of the cytopathic effect (CPE) of *S. typhosa* on J-111 cells were made in stained coverslip preparations from 10 experiments. The cellular alterations included retraction and vacuolation of cytoplasm, cytoplasmic and nuclear dissolution, increased basophilia, pyknosis of the nucleus, and rounding of cells or their detachment from the glass. The effects at varying periods of infection are illustrated in figure 1A to F. At 30 minutes the cells looked normal. With time CPE increased until at 50 to 74 hours destruction of the monolayer culture was complete. During the early stages of infection the cytoplasm of the infected cell appeared granular and contained small vacuoles with few to many bacteria within them (figure 1B). At times a cell was observed to contain long filamentous bacilli wrapped around the nucleus,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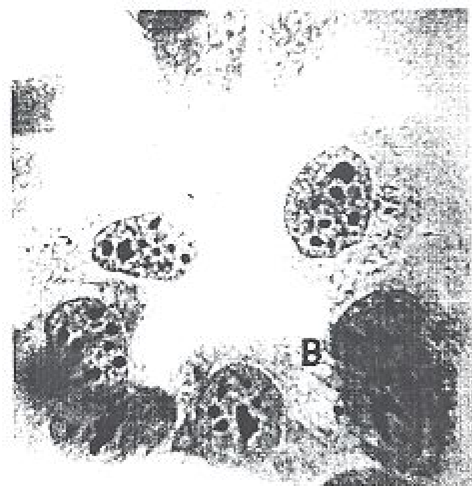
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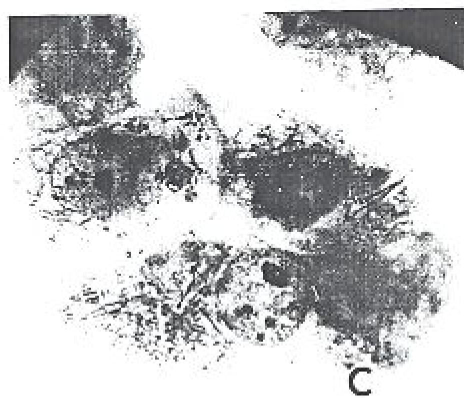
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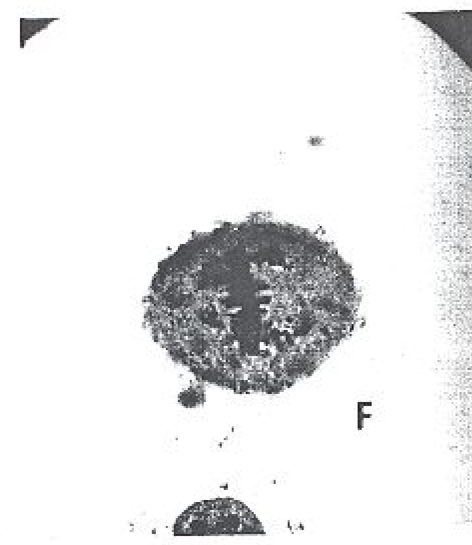
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and they stained darker than the regular bacillary forms (figure 1C). In the later stages of infection these filaments were observed in association with spherical forms stained like typhoid bacilli. Most of the cells still were attached to the glass and were completely filled with bacteria (figure 1D). The cytoplasm of the infected cells was lightly and unevenly stained, while the nucleus was dark, wrinkled, and displaced toward the margin of the cell. In the final stages of infection bacteria were contained within huge vacuoles which occupied most of the cytoplasm (figure 1D and E). At this time cells varied markedly in size and shape, nuclear fragments and cell debris were seen floating in the medium, and more than 80% of the original cells of the monolayer had left the glass.

It was surprising to note that a number of cells undergoing mitosis contained actively multiplying typhoid bacilli. Cells in all stages of division contained bacteria after 12 hours incubation. A cell in metaphase and containing bacteria is shown in figure 1F.

Development of Spheroplasts in Intracellular S. typhosa

In the attempt to study the fate of *S. typhosa* following phagocytosis intracellular spherical bodies were observed in *S. typhosa*-infected cells early in the the experimental work. This was interpreted as a host response to the intracellularly multiplying bacteria.

To elucidate their nature further a study was made of these spherical forms in 25 different experiments. Stained coverslip preparations of typhoid-infected J-111 cultures were examined for the presence or absence of intracellular spherical bodies at different periods of infection. At least 40 coverslip preparations were examined in each experiment, and spherical forms were found in 15 of the 25 experiments.

The photomicrographs in figure 2 illustrate observations made during the course of infection. Spherical bodies were never seen before the 6th hour of incubation but frequently were present after 12 hours. Photomicrographs 2A and B illustrate the increase in numbers of spherical forms as well as bacillary forms during the period from 12 to 24 hours; they illustrate also the location of spherical forms within vacuoles. Filamentous forms, seen in 2B, usually were detected earlier than spherical bodies.

Besides the filaments, other pleomorphic forms not shown in the photographs were sometimes observed to be associated with the spherical forms. Late in infection, after 48 to 72 hours, a cell might be completely filled with spherical bodies contained within a few large vacuoles (2C and D) or even within 1 very large vacuole (2E). Also, the vacuoles impinged against the cell nucleus, distorting and displacing it. Occasionally, cells containing vacuoles filled with rounded forms were observed to have burst, and spherical bodies were being extruded into the extracellular fluids, as shown in 2F.

The possibility that these sphero-

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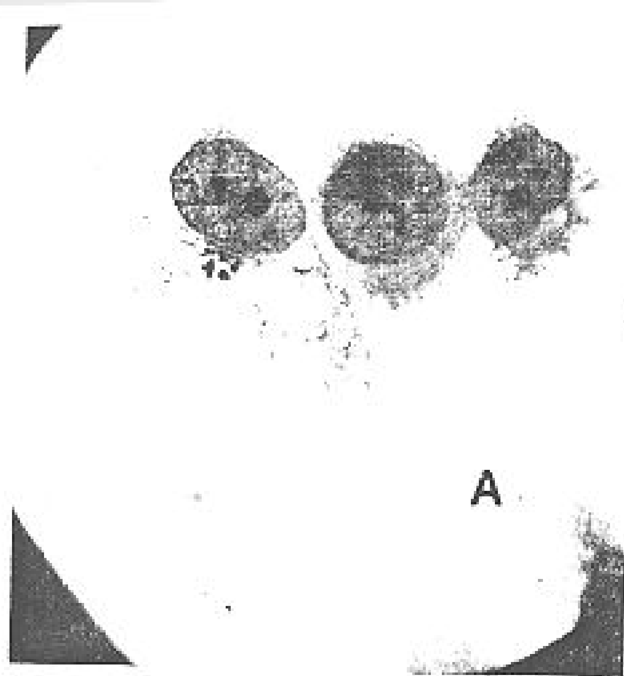
FIGURE 1.—Cytopathic effects of intracellular *Salmonella typhosa* in J-111 cells at various times after contact between bacteria and cells. May-Gruenwald-Giemsa stain. Magnification is 875× except for A which is 350×. A.—At 30 minutes no cytopathic effects were observed. B.—At 8 hours a few bacilli are within vacuoles. C.—Filaments are associated with bacillary forms at 24 hours. D.—At 48 hours the figure shows a bacteria-filled vacuole and displaced nuclei. E.—At 72 hours there are huge vacuoles filled with bacteria. F.—Cell in metaphase contains many bacilli (at 24 hours).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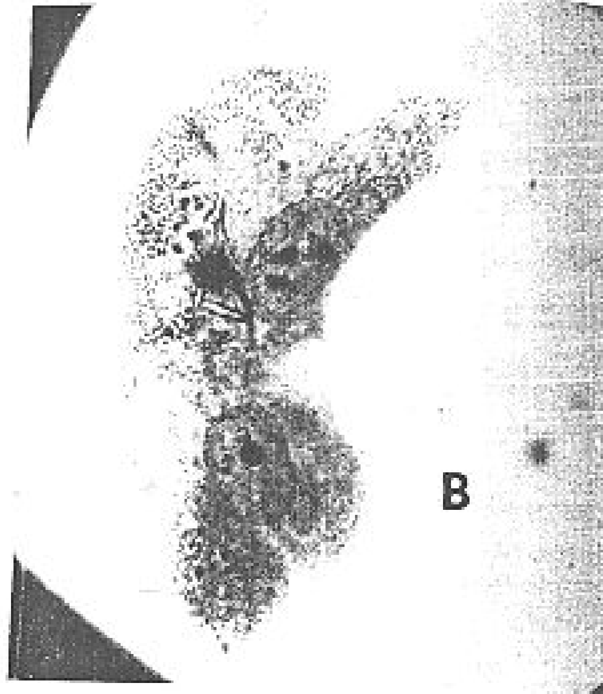
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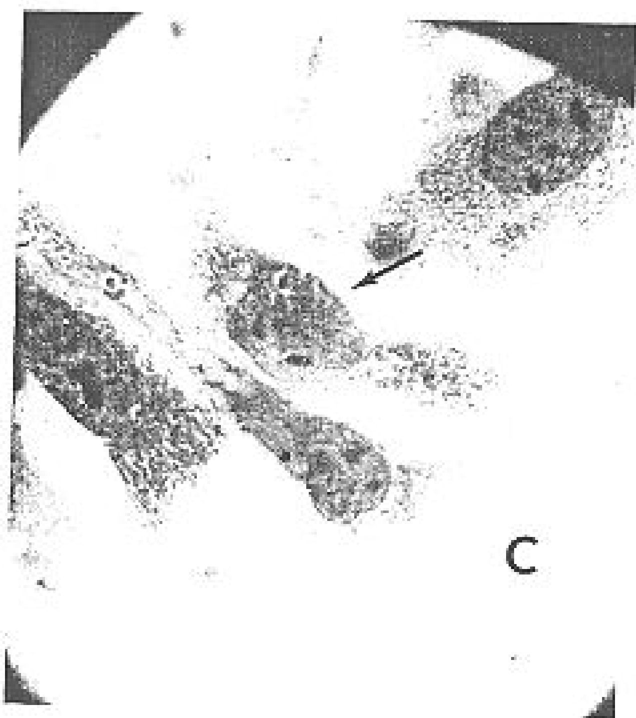
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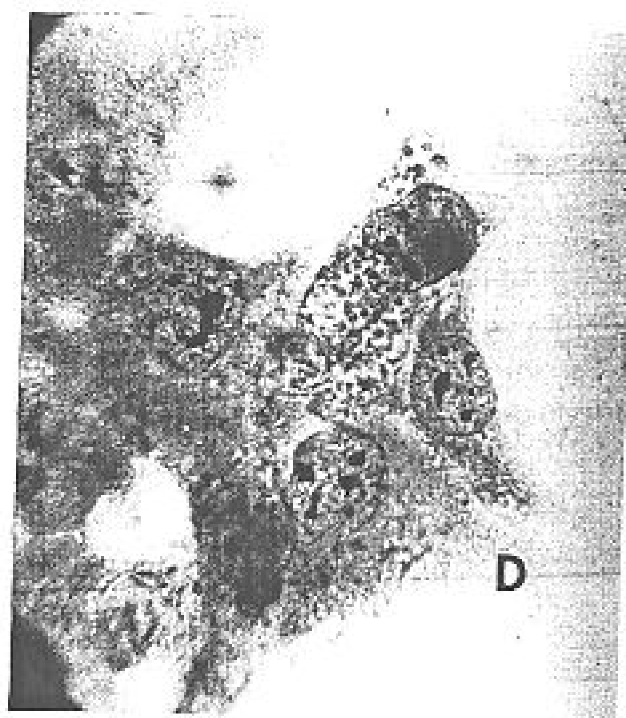
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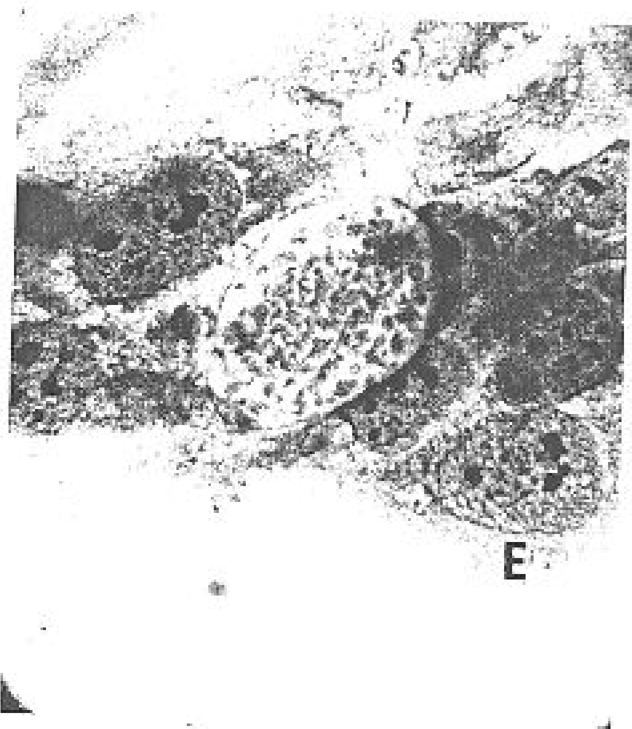
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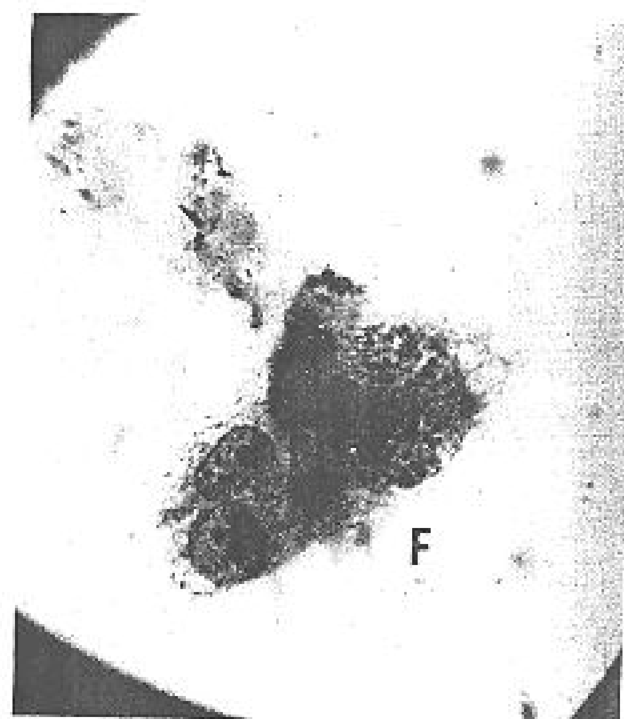
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TABLE 1.—Intracellular spherical bodies in J-111 cultures inoculated with *Salmonella typhosa*

Hours after inoculation	Number of J-111 cells containing the indicated number of spherical forms*				Indices of intracellular spherical bodies‡
	Range†: 0	1–5	6–20	21–40	
	Factor: 0	1	3	8	
0.5	200 (0)	0 (0)	0 (0)	0 (0)	0
7	197	3 (3)	0 (0)	0 (0)	3
17	130	50 (50)	19 (57)	2 (16)	123
26	131	49 (49)	20 (60)	0 (0)	109
50	140	37 (37)	23 (69)	1 (8)	114

* 200 cells counted.
† Number of spherical bodies in cells.
‡ The index for each test period is the sum of the products; the product in each range (figure in parentheses) is the number of cells × the weighting factor. See *Methods*.

plasts were due solely to the action of penicillin was considered. To rule this out the following tests were done. Suspensions of *S. typhosa* were added to tissue culture medium (EBM-D), to EBM-D containing penicillin and streptomycin, and to EBM-D containing the antibiotics plus sucrose and magnesium ions, respectively. The suspensions were incubated at 37 C, and at subsequent intervals (0.5, 1, 2, 4, 6, 12 and 24 hours) microscopic observations of Gram-stained smears of each of the suspensions, prepared as described by Amano et al (1956), did not reveal the presence of spheroplasts. In another group of tests suspensions of *S. typhosa* previously treated with penicillin and streptomycin for 6 hours were added to monolayer coverslip cultures of J-111 cells in Leighton tubes as previously described. At appropriate intervals after the addition of bacteria coverslips were removed and stained as already described. At no time were spheroplasts observed in the J-111 cells or in the extracellular fluid. Only a very few intracellular bacilli were seen. In stained preparations of monolayer cultures of monocytes infected with untreated bacilli serving as controls certain individual J-111 cells were completely filled with spheroplasts, whereas adjacent cells contained none, even though they were filled with bacilli. If the spheroplasts had been formed extracellularly, induced possibly by penicillin, and subsequently phagocytized, one would expect to find intracellular spheroplasts distributed among the cells with some uniformity.

Quantitative estimate of the spherical forms.—Since it appeared that the intracellular formation of the spherical bodies could be interpreted as a cell response to bacterial infection of the monocytes, stained preparations were examined for evidence that the spherical forms increased in numbers as infection progressed. A quantitative estimate of the increase in numbers of the intracellular spherical forms was made as described in *Methods*. The treatment and tabulation of the data in table 1 illustrate the procedure used in this and

FIGURE 2.—Spherical bodies in J-111 cells after incubation of cells with *Salmonella typhosa*. May-Gruenwald-Giemsa stain; magnification 875× except for E which is 1750×. A.—A few spherical forms within vacuoles after 12 hours. B.—Larger number of spherical bodies in association with bacillary and filamentous forms after 24 hours. C.—Numerous spherical bodies within cells after 48 hours (arrow indicates a vacuole completely filled with these spherical forms). D.—Several filled vacuoles after 72 hours. E.—From preparation D but with higher magnification. F.—A vacuole filled with spherical forms has burst.

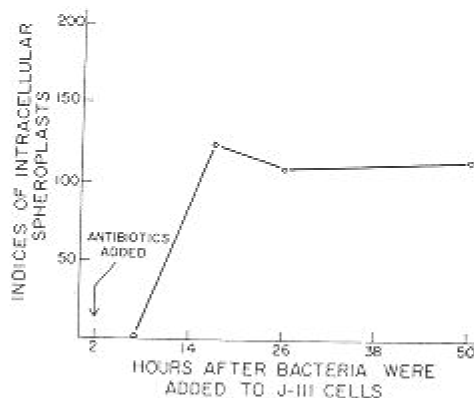

FIGURE 3.—Increase in numbers of spheroplast-like forms in *Salmonella typhosa*-infected J-111 cell cultures.

subsequent experiments. Each recorded count is an average of 2, and the 2 differed by only a few spherical forms. In figure 3 the relative numbers of intracellular spherical forms, expressed as "indices of intracellular spherical bodies," were plotted against hours of incubation. A few spherical forms appeared in some cells 7 hours after their inoculation with *S. typhosa*. The curve indicates an appreciable increase in numbers of the spherical forms at 17 hours, after which the number remained unchanged. In the interpretation of the curve it is pointed out that by 24 hours many of the cells filled with spherical bodies had degenerated and therefore would not be counted. This explains the leveling off of the curve after 24 hours. Since the results in this experiment were based on stained preparations, further tests were needed to determine the viability of the spherical bodies.

Tests for viability of the intracellular spherical forms.—Since it now seemed possible that the spherical bodies observed in the *S. typhosa*-infected cells were viable spheroplasts, a special medium was employed for the purpose of maintaining their morphological integrity. The sucrose and magnesium ion-supplemented medium used was a modification of one utilized by Weibull (1953) and Lederberg and St. Clair (1958).

Two different media were used for comparative colony counts of disrupted *S. typhosa*-infected cells containing spherical forms; unsupplemented heart infusion agar (Difco Laboratories, Detroit, Michigan) and the same medium supplemented with 17 g of sucrose per 100 ml of agar and 10 ml of a previously autoclaved 20% solution of magnesium sulfate ($MgSO_4 \cdot 7H_2O$) per liter. The agar was poured into Petri plates and stored at 4 to 6 C until needed.

Before plating the suspensions of disrupted J-111 cells onto unsupplemented and supplemented media for comparative colony counts a known suspension of the regular bacillary form of *S. typhosa* was plated on each medium. After 24 hours incubation at 37 C no difference was noted in the numbers of colonies on the 2 media, nor was there any observed difference in morphology of the colonies or of the stained individual bacilli. It would follow, therefore, that, if a defined inoculum of disrupted cells containing both regular bacilli and spheroplast-like forms resulted in a larger number of colonies on the spheroplast medium than on unsupplemented agar, a part of the colonies probably were derived from spheroplasts. Accordingly, the following experiment was done.

Infected cultures of J-111 cells were divided into 2 groups, A and B, depending on the plan for subsequent treatment. After incubation at 37 C for 2 hours all tubes were taken out of the incubator, the cells were washed to remove residual bacteria, and the fluid was replaced with fresh tissue culture medium. In order to prevent multiplication of residual extracellular bacilli, as previously explained, the fresh medium

added to each cell culture contained 40 units of penicillin and 40 μg of streptomycin per ml. All cultures were returned to the incubator and after 6, 26 and 48 hours, respectively, duplicate tubes from each group were removed and the medium from each tube was discarded. The infected cultures were then subjected to the following procedure: After washing twice with BSS 0.6 ml of a 0.05% trypsin solution were added to each tube, and the tubes were incubated at room temperature (25 to 30 C) for 5 minutes. After trypsinization 1.4 ml EBM without serum or antibiotics were added to cell suspension A to bring the final volume to 2 ml. Cell suspension B was likewise diluted to 2 ml with the same medium supplemented with sucrose and magnesium ions. Each diluted suspension was then transferred to a Teflon Potter-Elvehjem tissue grinder, and the cells were disrupted by homogenizing at 1500 rpm in a variable speed stirrer. Cell-bacterial suspension A was removed, diluted by 10-fold increments with EBM without serum or antibiotics and 0.1 ml of each dilution plated out in duplicate on heart infusion agar. Cell-bacterial suspension B underwent the same treatment except that the diluting fluid was supplemented with sucrose. Each dilution was plated out in duplicate on spheroplast medium.

The colonies appearing after 24 hours incubation of the agar plates were counted, and the total numbers of bacteria in the original infected cell culture were calculated. The data are shown graphically in figure 4. More bacterial colonies grew on the spheroplast medium (curve B) than on the unsupplemented agar (curve A). Furthermore, at 8, 28 and 50 hours, respectively, there were approximately 5, 3 and 1.3 times as many colonies on the first medium as on the second. Curve C, the difference in the numbers of colonies on the 2 media, shows a plateau which indicates no marked changes in the numbers of spherical forms. This is consistent with the curve in figure 3, which shows a leveling off in the increase of intracellular spherical bodies after 16 hours.

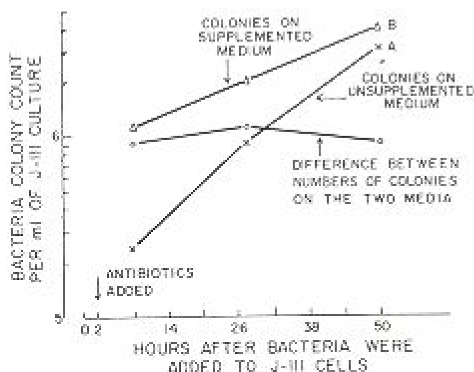

FIGURE 4.—Numbers of bacterial colonies on supplemented and unsupplemented media inoculated with *Salmonella typhosa*-infected J-111 cells disrupted at various times. The infected cells contained spheroplast-like forms.

The colonies growing on the 2 kinds of media were indistinguishable from each other grossly. They were white, smooth, circular colonies with even edges and measured 0.5 to 1.5 mm in diameter. On the other hand, microscopic observations of Gram-stained smears prepared as described by Amano et al (1956) revealed differences as illustrated in photo micrographs A to D in figure 5. In 5A the organisms from colonies growing on the unsupplemented medium were Gram-negative bacilli of relatively uniform size and shape and predominantly homogeneous. In contrast, organisms from colonies growing on the supplemented medium (5B, C and D) appeared markedly pleomorphic, with many filamentous and club-shaped forms. In the same photomicrographs are many spherical bodies of different sizes, resembling spheroplasts. All organisms observed were Gram-negative.

It was pointed out that the number of

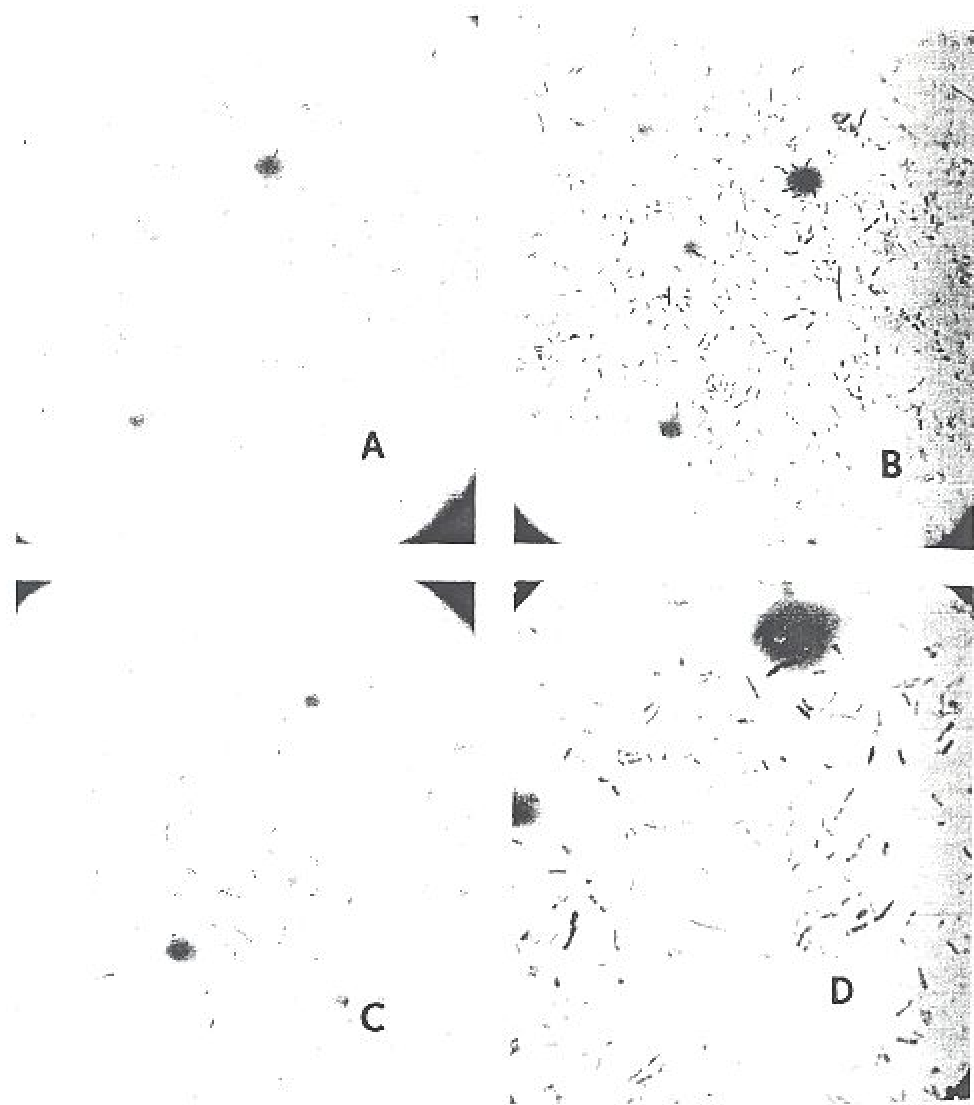

FIGURE 5.—Microscopic characteristics of *Salmonella typhosa* after passage through J-111 cells. Gram stain, Amano; magnification 875× except for D which is 1750×. A.—Bacteria from colony on unsupplemented medium. B.—Bacteria from colony on supplemented medium; pleomorphism illustrated by filamentous and spherical forms. C.—Spherical forms predominate in preparation from colony on supplemented medium. D.—Pleomorphic forms including spherical bodies.

colonies on the supplemented medium was considerably greater than on unsupplemented. Also, many of the colonies on the supplemented medium were shown by stained preparations to contain large numbers of pleomorphic organisms, whereas this was not true for colonies on the same medium that were derived from regular cultures of *S. typhosa*.

The results with these experimental cultures supported the indications of the stained coverslip preparations and, together, these findings were accepted as evidence that the spherical bodies were spheroplasts.

Effect of Specific Antisera on Intracellular Infection

Since intracellular infection invariably led to changes in the host cell, the question arose whether the effects of the bacteria on the cell culture could be modified by use of specific antiserum. To answer this question, tests were carried out with a Vi antiserum, an O antiserum, and combined Vi and O antisera. The O antiserum was prepared against *S. typhosa* strain no. 63 from the Michigan Department of Health and the Vi antiserum against *S. ballerup* (now classified in the Bethesda-Ballerup group) by the procedures described by Edwards and Ewing (1955). The effect of an antiserum was judged by its influence on (1) uptake of bacteria, (2) intracellular multiplication, and (3) cytopathology of host cells. Bacteria seen within cells during the first 30 minutes of infection were interpreted as evidence of ingestion or phagocytosis, while those observed subsequently were considered multiplication. The quantitative estimate of intracellular bacteria in the J-111 cells under test was based on examination of stained coverslip preparations, as described previously. For measuring the cytopathic effect (CPE) in variously treated cell cultures 40 to 50 fields of each stained coverslip preparation were examined under 100× and 400× magnification, and an estimate was made of the total cell population exhibiting degeneration by noting the degree of CPE in comparison with the control in each particular experiment. The estimates on 2 preparations were averaged to the nearest 5%, and the relation between test and control cells was expressed as a ratio. In each experiment, also, tubes of cells containing no antiserum or bacteria were included as an additional control of the normal behavior of cells alone.

General experimental procedure.—To test the effect of an antiserum on the progress of infection of J-111 cells in contact with *S. typhosa* the following procedure was used. Leighton tubes containing 3-day-old cultures of J-111 cells were arranged in 4 groups of 10 tubes each. All cultures were kept at 37 C except during manipulation procedures. To each tube 0.1 ml of antiserum diluted 1:10 was added to groups I, II and III, respectively, at 8 hours before, at the same time as, and 8 hours after addition of the infecting dose of *S. typhosa*. Preimmunization serum was added to control group IV at 8 hours prior to infection. Before the cell cultures were inoculated with bacteria EBM–H was replaced by EBM–D after cells had been washed with BSS. Serum lost during the process of washing (groups I and IV) was not replaced at this time. All cell cultures were inoculated with a standardized dose of *S. typhosa* at approximately the same time. Two hours later the extracellular fluids were withdrawn, the cultures washed twice, and fresh EBM–D containing 40 units of penicillin and 40 μg of streptomycin per ml was added. Serum was now replaced in tubes in which it had been lost during the process of washing (groups I, II and IV). All cultures were returned to the incubator and at different times thereafter duplicate tubes were removed and the coverslips fixed, stained, and studied. The numbers were determined microscopically, as described under *Methods*.

Results with Vi antiserum.—The lack of a measurable effect of Vi antiserum on intracellular multiplication is shown by the summarized results expressed graphically in figure 6. Comparison of the curves shows that during the first 6 hours the numbers of intracellular bacilli increased at a similar rate in 3 of the 4 groups (curves II, III and IV), while in the other (curve I) intracellular bacterial multiplication appeared to be

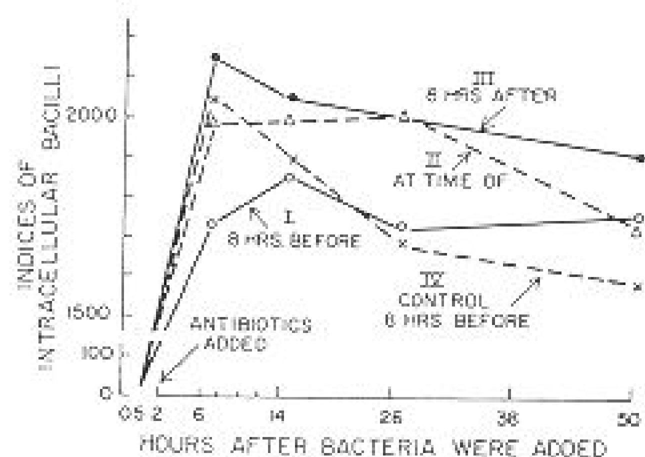

FIGURE 6.—Effect on intracellular growth of *Salmonella typhosa* when Vi antiserum is added to J-111 cells before, at the same time as, and after the addition of the bacteria.

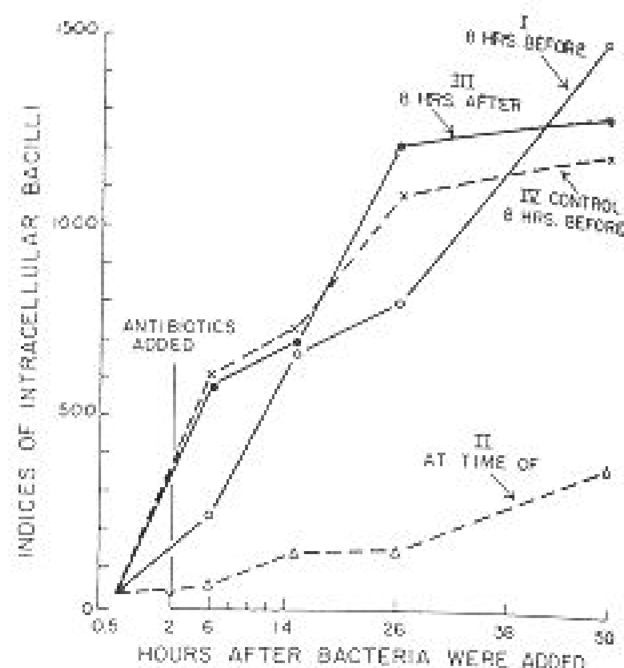

FIGURE 7.—Effect on intracellular growth of *Salmonella typhosa* when O antiserum is added to J-111 cells before, at the same time as, and after the addition of the bacteria; and preimmunization (control) serum 8 hours before.

slightly suppressed. Following their initial increase the number of bacilli in all 4 groups varied slightly for the remainder of the experiment due to filling of the cells with bacteria and consequent detachment from the glass.

The influence of Vi antiserum on the cytopathic effect of *S. typhosa* is indicated by the results in table 2. During the first 6 to 14 hours incubation there was evidence of delay of cell degeneration in the group of cells in which Vi antiserum had been added before the typhoid bacilli and also in the group in which antiserum and bacilli had been added at the same time. Thus the cytopathogenic effect generally was very mild in the test cultures by the first 6 hours, indicated by the bold face ratios which point out where the greatest difference occurred between control and test cells. CPE increased slightly by 14 hours and rapidly thereafter, as shown by the correlation between decreasing ratios and increasing time intervals. The group of cells which received Vi antiserum 8

TABLE 2.—The effect of specific antiserum on the cytopathologic effect of *Salmonella typhosa* in J-111 cells

Kind of antiserum	Addition of antiserum related to addition of bacteria	Per cent of control cells* showing CPE/per cent of test cells				
		Hours after addition of bacteria:				
		0.5	6	14	26	50
Vi	8 hours before	Negative	40/15† 2.7	40/25 1.6	80/65 1.2	100/90 1.1
	At same time	Negative	40/20 2.0	40/20 2.0	80/55 1.5	100/90 1.1
	8 hours after	(Negative)	40/(25)	40/40 1.0	80/80 1.0	100/100 1.0
O	8 hours before	Negative	Negative	15/10 1.5	45/15 3.0	75/55 1.4
	At same time	Negative	Negative	15/15 1.0	45/15 3.0	75/65 1.2
	8 hours after	(Negative)	(Negative)	15/15 1.0	45/25 1.8	75/40 1.9
Vi+O	8 hours before	Negative	Negative	30/15 2.0	30/20 1.5	75/50 1.5
	At same time	Negative	Negative	30/0 ∞	30/30 1.0	75/50 1.5
	8 hours after	(Negative)	(Negative)	30/20 1.5	30/20 1.5	75/30 2.5

Negative indicates the lack of observable CPE. Parentheses are a reminder that antiserum had not yet been added. In these instances a ratio has no meaning and is omitted. The bold face numbers point out the ratios showing the greatest differences between control and test cells.

* Control cells had been treated with preimmunization serum 8 hours before addition of bacteria.

† 40/15 = 2.7.

hours after addition of bacteria showed little delay of cell degeneration, and CPE generally was intense by 14 hours incubation, i.e., no difference between control and test cells.

Results with O antiserum.—The results expressed graphically in figure 7 show the estimated numbers of intracellular bacteria in J-111 cells in groups I, II and III, respectively, which received O antiserum before, at the same time as, and after addition of *S. typhosa*, and in control group IV which received preimmunization serum 8 hours before the bacteria were added. All groups of infected cell cultures contained relatively the same numbers of intracellular bacilli at 30 minutes, indicating that the uptake of typhoid bacilli was not prevented in any of the 4 groups. Thereafter, the numbers of intracellular bacilli increased rapidly in the cells of control group IV and of groups I and III, but intracellular bacteria were less numerous in cells of group II. At 26 hours the number of bacteria in group II was still low compared with the numbers in the other 3 groups. When the experiment ended at 50 hours, there was a marked difference in the number of intracellular bacilli in group II in comparison with the number in any of the other groups.

The data summarized in table 2 indicate that O antiserum was less effective in preventing cytopathology in the infected cells than was Vi antiserum. In this set of experiments degenerative changes did not appear in any of the test or control groups of cells until after 6 hours of incubation. By 14 hours degenerative changes had appeared in all groups of infected cells. However, there was a slight difference between control and test cells in the group which received O antiserum before addition of typhoid bacilli. There was no difference between control and test cells in the groups in which antiserum was added together with and after bacteria. On the other hand, at 26 hours incubation an apparent protective effect of the O antiserum was noted in the group of cells in which O antiserum was added before bacteria and also in the group in which antiserum and bacteria were added at the same time; that is, the greatest difference between control and test cells appeared at 26 hours. Subsequently CPE became intense for both groups as evidenced by the abrupt lowering in the ratios. When antiserum was added to cells after the addition of the typhoid bacilli, no difference in CPE between control and test cells was noted at 14 hours. But by 26 hours and at 50 hours when the experiment ended, although the test group of cells showed an increase in the number of degenerating cells, the ratios suggest some protective effect of O antiserum. Summarizing, specific O antiserum added to J-111 cells at the time of inoculation with *S. typhosa* suppressed considerably the intracellular multiplication of bacteria but, on the other hand, gave equivocal results regarding delay or prevention of cytopathic changes in the infected cells.

Results with combined Vi and O antisera.—To test the combined effect of Vi and O antisera cultures of J-111 cells were arranged in the usual 4 groups of 10 tubes each. The test dose of 0.1 ml combined antisera contained 0.05 ml each of Vi and O antisera diluted 1:10.

As shown in figure 8, uptake of typhoid organisms was not prevented in any of the 4 groups, as judged by the numbers of intracellular bacilli at 30 minutes. At later times the number of intracellular bacteria in group II which received the combined antisera at the same time the organisms were added was much smaller than in the other 3 groups.

The observations recorded in table 2

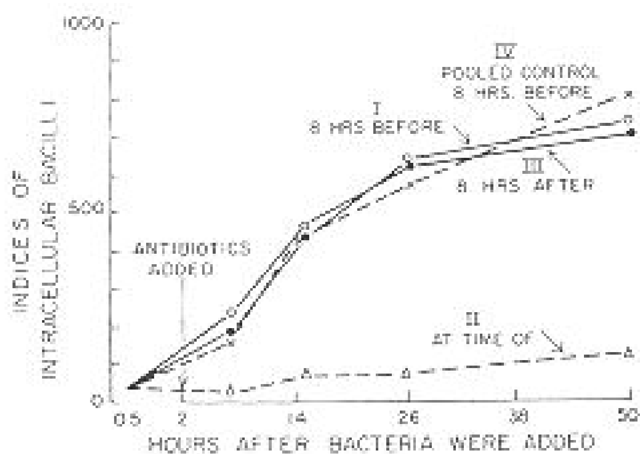

FIGURE 8.—Effect on intracellular growth of *Salmonella typhosa* when combined Vi and O antisera were added to J-111 cells before, at the same time as, and after addition of bacteria; and pooled preimmunization (control) serum 8 hours before.

indicate the relative degree of cytopathology of *S. typhosa*-infected cells in the presence of the combined Vi and O antisera added at different times. Morphologic changes were not observed in any group until after 14 hours of incubation. At that time no cytopathology was observed in the cells which had received the antisera at the same time as the bacteria. Also, there was only a mild cytopathogenic effect in the cells which received the antisera before the bacteria were added, suggesting a protective effect. However, the cells which had received the antisera after addition of the bacteria showed marked CPE and therefore gave little evidence of protection. The experiment demonstrated that combined Vi and O antisera added to J-111 cells at the same time as *S. typhosa* markedly suppressed intracellular multiplication of bacilli and for at least 14 hours prevented cytopathic changes in the infected cells. In another separate experiment the results were confirmed as to relative numbers of intracellular bacteria following inoculation of J-111 cell cultures along with combined Vi and O antisera in comparison with Vi and O antisera used separately.

DISCUSSION

Bacterial infection of tissue cells in culture involves ingestion of living bacteria. The end result is determined by the character of interaction between cells and bacteria. This interaction may result in alteration either of the parasite, the host cell, or both; the outcome depends on multiple factors. We recognize, for example, that a critical condition for maintenance of the bacterial parasite within the host cell is the presence of a suitable nutritional environment. But for the most part, as pointed out by Dubos in 1954, we still must admit our ignorance of the factors within host cells which may, on the one hand, bring about rapid death of certain microorganisms and, on the other, allow them to survive and proliferate.

The interactions between host cells and bacterial parasites may be expressed in a number of ways. At times, the host cells rapidly kill the bacteria which they engulf, as happens with blood leukocytes and *Diplococcus pneumoniae*; sometimes the parasite is able to survive but not multiply, as with *Mycobacterium smegmatis*; at still other times, the bacterium survives and multiplies within the cell, as with *Brucella abortus*, *S. typhosa*, and others.

In the present study typhoid organisms not only multiplied readily within the J-111 cells, but they were changed by cell passage. The most striking change was the development of spherical forms accepted as spheroplasts within infected cells, usually observed after 6 to 8 hours incubation and preceded by the appearance of long, slender, filamentous forms. The bases for acceptance of the spherical forms as spheroplasts were considered under *Results* and took into consideration the generally accepted definition of spheroplasts as expressed, for example, by Lamanna and Mallete (1965). The resistance of

these forms to the grinding treatment used in the experimental procedure suggests less fragility than is usually attributed to protoplasts. Perhaps an explanation can be found in the amount of cell wall remaining on the spheroplasts. The discussion by McQuillen (1960) is relevant.

To explain the origin of the spheroplasts and the pleomorphic forms which preceded them there are 2 possibilities: they may have been produced outside the cell and subsequently ingested, or they may have developed as a result of intracellular events. The formation of spheroplasts extracellularly or in cell-free media has been described by several authors. Michael and Braun (1959) reported the conversion of *Shigella dysenteriae* into spheroplasts. Showacre et al (1961), Hopps et al (1961), and Smadel (1963) observed the formation of *S. typhosa* spheroplasts from extracellular bacteria but not from bacteria inside healthy tissue cells. When typhoid-infected mouse fibroblasts were treated with penicillin, spheroplast formation of bacilli was induced only in extracellular bacilli or in dead or dying cells containing organisms. Landman et al (1958) described methods involving defined media for the conversion by penicillin of *Proteus mirabilis* and *Escherichia coli* to the spheroplast form and thence to the L form. Lederberg and St. Clair (1958) and others showed that penicillin-inhibited growth of *Proteus vulgaris* and *Escherichia coli* led to protoplast formation of these organisms in vitro. Lysis of these osmotically fragile spheres could be forestalled by growing them in a protective hypertonic medium containing sucrose and magnesium ions. It has been suggested that penicillin causes inhibition of synthesis of cell wall material (Park and Strominger, 1957), leading to eventual lysis of the bacterium unless forestalled by incubating the penicillin-treated culture in a protective hypertonic medium. During the present study attempts to produce spheroplasts extracellularly in the J-111 cell-bacterial system were unsuccessful.

A possible explanation of intracellular formation of spheroplasts is that conditions in the infected cell interfere with normal synthesis of bacterial cell wall constituents and proliferation then leads to spheroplast production. Or, perhaps, enzymatic degradation of a part or all of the bacterial cell wall results in conversion of ingested bacteria into spheroplasts or protoplasts. On the other hand, the possibility of penicillin-induced spheroplasts inside cells cannot be excluded, especially in the light of reports that some antibiotics can enter tissue cells in vitro. Eagle (1954) reported that penicillin penetrates tissue cells in vitro. Showacre et al (1961) demonstrated by phase microscopy that several antibiotics, including penicillin, penetrated mammalian tissue cells rapidly and retained their biologic properties inside the cells. Richardson and Holt (1962) found that streptomycin acted synergistically with penicillin to inhibit the growth of *Brucella abortus* within bovine cells cultured in vitro, thus demonstrating that these antibiotics entered the tissue cells in sufficient concentration to affect intracellular bacterial growth. In the present study intracellular multiplication of typhoid bacilli was not inhibited when penicillin and streptomycin were added to the extracellular fluid of the host-cell bacterial system. It might be argued that a concentration of these antibiotics could be attained in a semipermeable cell such that, although inadequate for bactericidal action, it would be sufficient to induce spheroplast formation. The observation by Richardson and Holt (1962) on the building up of a concentration of penicillin in the

presence of streptomycin may be relevant. The possibility of such a situation in which the increased concentration of penicillin might induce intracellular spheroplasts needs to be kept in mind. More refined experiments need to be designed to obtain definitive information on this point. In favor of their intracellular formation is the fact that spheroplasts were never observed before 6 hours of incubation and that they were scarce then; only after longer periods were larger numbers observed.

Based on the experimental results and on the discussion above, it is suggested that in these experiments the typhoid organism went through a series of steps in its conversion to a spheroplast, namely, from bacillus to filamentous or other pleomorphic form, and then to spheroplast. This series of events is most readily explained on the basis of inhibition of cell wall synthesis. Thus the first evidence of inhibition would be a failure of cross wall synthesis, resulting in filament formation. Then continued interference would result in production of spheroplasts. This reasoning appears consistent with the work of Lederberg and St. Clair (1958), among others. Fine structure analysis by electron microscopy could be expected to help elucidate this problem.

The intracellular formation and survival of spheroplasts has possible implications in the pathogenesis of disease and may be related to the concept of microbial change in response to environmental conditions. Guze and Kalmanson (1964a, b) observed that bacteria persisted in "protoplast" form in rat kidneys after treatment of experimental enterococcal pyelonephritis with penicillin, prompting speculation concerning the possible role of these forms in the pathogenesis of chronic pyelonephritis and frequent recurrence of renal infection in man. Dwarf forms of staphylococci and L forms of many organisms may be cited as examples. L type forms have been incriminated in various disease processes and have involved different species of organisms and different species of hosts. The possible relationship of these various forms to inapparent infections was recognized by McDermott (1959) who regards latent infections as an extreme form of microbial adaptation accompanied by morphological changes of the parasite. Lederberg and St. Clair (1958) and Klieneberger-Nobel (1960) have suggested that spheroplasts may be intermediate to conversion from bacillary to stable L forms. In this study the interesting question arises whether formation of spheroplasts may be one way of maintaining the typhoid carrier state. The inference seems reasonable in the light of Guze and Kalmanson's experiments and McDermott's thesis of latent infections. Laboratory workers know how difficult it is to isolate typhoid organisms from carrier specimens; at times it is not possible to recover organisms at all. In this area of particular concern to the public health laboratory perhaps consideration should be given to devising media and techniques which would allow the isolation of spheroplasts or the L form of *S. typhosa*, when present, and would make possible studies of the significance of such forms in relation to the human disease.

The capacity of specific Vi and O antisera to affect bacterial multiplication and to delay or modify cellular degeneration of J-111 cells following their parasitization with *S. typhosa* suggests that the course of infection may be influenced considerably by the kind of antiserum present during the interaction of bacteria and cells, the size of the antiserum dose, and the time of addition of bacteria in relation to the addition of serum. The protective effect of Vi anti-

serum observed in the interaction of *S. typhosa* and J-111 cells occurred promptly, as shown in table 2. Cellular degeneration of the typhoid-infected cells was effectively delayed early in the infection cycle and was more pronounced when the antiserum was given before the addition of bacteria. However, the protective effect was gradually lost, and little protection was observed when the antiserum was added 8 hours after the cells were infected. On the other hand, the role of specific O antiserum on cellular degeneration seems questionable under the conditions of the experiments. It is not possible to state unequivocally that O antiserum also confers protection by diminishing or attenuating the CPE of the typhoid-infected cells. An apparently high protective effect which did not persist was observed midway in the infection cycle (table 2), although little or none was seen earlier. No immediate explanation is available for this observation other than to suggest that perhaps the main suppressive effect of O antiserum is on bacterial multiplication, while a secondary effect is directed against CPE late in infection.

Combined Vi and O antisera inhibited the progress of infection in terms of both bacterial multiplication and cytopathology of the J-111 cells. Even when the antisera were given after infection of the cells, a certain degree of protection against CPE was observed. The observation that combined Vi and O antisera affects bacterial multiplication as well as cytopathology raises a number of questions concerning mechanisms. For example, does the antiserum enter the cell and exert its effect intracellularly, or does it combine with the bacteria extracellularly before they are phagocytized? Gelzer and Suter (1959), working with *S. typhimurium* and rabbit monocytes, concluded that antibody entered the cells only in combination with bacteria during phagocytosis. Simple passage of the serum through the membranes of the living cells is not supported by presently available data. The hypothesis that the antiserum acts on the extracellular bacteria before they are ingested receives some support from the experiments on the effect of time of addition of antiserum to the test system. When antibody was added following infection, there was no appreciable effect on the intracellular multiplication of the bacteria but, when it was allowed to act on the bacteria prior to their entry into the cells, there was a marked effect.

There are several possible explanations for the minimal effect of Vi antiserum on multiplication and its suppressive effect on cellular pathology. It may be that Vi antibody combines with and neutralizes soluble Vi antigen or perhaps the antibody is kept away from the vital part of the bacterial cell purely by the thickness of the Vi layer, as suggested by Nagington (1956). There is the possibility, too, that Vi antibody may inhibit the V forms of the culture while allowing the nontoxic W forms to grow.

An explanation of the suppressive effect of O antiserum on bacterial multiplication may be that, although it has minimal effect on cytopathology, O antibody directs its action against the somatic antigen through flaws in the Vi coat, thus inactivating part of the bacterial population and keeping the numbers of organisms at a low level. Also, in the interaction between bacteria and host cells some of the Vi antigen may be degraded, exposing vital somatic parts on the bacterial surface sensitive to the O antibodies. It must be considered, too, that the typhoid culture might be a mixture of V and W forms. The O antibodies would then inactivate the W forms, perhaps leaving a few V forms which do not increase rapidly; this might explain

the feebly protective action of O antibodies against the cytopathic effects. The latter view is supported by the observations in the experiment with the combined Vi and O antisera in which, in spite of a low bacterial count in group II (figure 8), a relatively high cytopathic effect was noted at 26 hours of infection (table 2). Considering that the typhoid culture is a mixture of V and W forms, it is suggested that O antibodies in the mixture inactivated most of the W forms while at the same time the toxic V forms were being affected by the Vi antibody present. Since the effective test dose of combined antisera employed in this particular experiment (0.05 ml Vi and 0.05 ml O) was one-half of the dose used in the single antiserum tests (0.1 ml Vi and 0.1 ml O), it is speculated that probably not all of the bacterial forms were inactivated, although any that remained did not increase rapidly. The suppressive effect of Vi antibody on cellular degeneration likewise had been diminished because of the insufficient antibody concentration, and there were enough toxic V forms remaining to cause the relatively marked cytopathic effect observed late in the infection cycle. It would be informative, in future experiments, to determine if graded cytopathic effects and graded bacterial counts could be correlated with varying proportions of Vi and O antisera in the combined antiserum. A repetition of these experiments with different strains of *S. typhosa* tested with other lots of antiserum and with antisera prepared in different animal species could be expected to give further information on the mechanism of the observed effects of specific antiserum. The use of purified antisera prepared by precipitation or fractionation of immune serum would perhaps make it possible to define more clearly the component of the antiserum that is involved in its effect on cellular pathology and bacterial multiplication.

In the interpretation of the present results it is remembered that the nature of the reactions is such that they defy strictly quantitative measurement. At times a difference in results interpreted to be qualitative may in fact be only quantitative. For example, in looking at the observed effects of combined Vi and O antisera on infected cells it is not possible at present to differentiate unequivocally between the quantitative and qualitative contribution of each component.

SUMMARY

Under defined experimental conditions human monocytic cells were infected with *Salmonella typhosa*. The numbers of intracellular bacteria increased progressively during the observation period, and degeneration of the infected cells likewise was progressive. However, infected tissue cells in various stages of division, containing many bacteria but showing little or no cytopathology, were observed repeatedly. Marked pleomorphism of the intracellular bacteria occurred with some frequency. Spheroplasts were observed within vacuoles in the cytoplasm of the monocytic cells. In controlled plate cultures of disrupted, infected cells on sucrose-supplemented and unsupplemented media the spheroplasts appeared to yield colonies of *S. typhosa* in which there were many pleomorphic and spherical forms. Combined Vi and O antisera inhibited the progress of infection in terms of both bacterial multiplication and cytopathology of the J-111 cells. The Vi antiserum appeared to have the greater suppressive effect on cellular degeneration, and O antiserum on bacterial multiplication. These effects were additive only, and no synergistic action was noted. The observations suggest that the J-111 human monocytic cell-*S. typhosa* system may be par-

ticularly suited for the study of host-parasite interactions.

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