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Phagocytosis by HeLa Cells and Their Susceptibility to Infection by Human Tubercle Bacilli.* (22043)

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In order for an infectious agent to multiply within its host cell it must, of course, enter the cell by passing through the cell boundary. Since this portion of the cell is relatively impermeable even to molecules of moderate size, the passage of a particle as large as a bacterium or virus might be expected to be an unusual process. In the case of some of the bacteriophages it is apparently necessary only for the nucleic acid portion of the viral particle to enter the cell, the structural material of head and tail remaining on the outside of the cell wall(1). Such dissociation of viral nucleic acid from protein may later be found also in the animal viruses, yet it seems certain that with some of the larger infectious agents which multiply intracellularly, such as the rickettsiae and the leprosy bacillus, that the infectious agent enters the cell in intact form. The mechanism by which the infectious agent passes the cell membrane would thus appear to be an important factor in determining whether or not a cell is susceptible, that is, whether the agent can multiply within the cell. One such mechanism by which the infectious agent could pass the cell membrane could be the engulfment of the particle by the host cell, and it is possible that many of the differences between the growth of bacterial and animal viruses depend upon this activity by animal cells. The engulfment of droplets (pinocytosis) has been observed in "phagocytic" cells and cells of malignant origin, including the HeLa cell, by Gey *et al.*(2) and the engulfment of particles (phagocytosis) has also been described for many animal cells. The categoric separation of cells into "phagocytes" and "non-phagocytes" is unfortunate in this connection, because the difference between cells such as macrophages and fibroblasts is in degree of engulfing activity, rather than presence or absence of this activity.

Experiments described below deal with HeLa cells and the relationship between their phagocytic activity and the culture medium placed on them. It is seen that when HeLa cells are made more phagocytic by the use of media containing certain horse serums, human tubercle bacilli quickly enter the cell where they then grow luxuriantly, even when the medium is subsequently changed to one containing human serum, in which phagocytosis is at a minimum. If one examines a HeLa cell culture a few days after it was infected with the tubercle bacilli, large increases in the amount of intracellular growth are observed to result from the use of suitable horse serum media during the first day of infection, so that in effect the HeLa cells have been rendered more susceptible to this infection.

Materials and methods. The HeLa cells were grown on cover slips in flat-sided tubes. The cultures of the HeLa cells were started from cultures in 400-ml square bottles, the growth being washed 3 times with BSS (Hanks' balanced salt solution without sodium bicarbonate) then pushed off with a rubber policeman into 5 ml of the growth medium of

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Syverton, Scherer, and Elwood(3). Cell clumps were broken up with bulb and pipette and the suspension transferred to a flask containing the remainder of the growth medium, 50-70 ml per bottle. The cells in growth medium were then distributed in 1.0-ml amounts into the test tubes. After 2 days the cover slip was washed twice in the tube with 1.0 ml of BSS, 1.0 ml of the experimental medium was introduced, and 0.05 ml of the bacterial suspension added. The bacterial suspension was prepared from 4-7 day cultures of the H37Rv strain of the human tubercle bacillus grown in Dubos media containing 0.05% Tween, from which the bacilli were sedimented once in the centrifuge and resuspended in BSS by several forceful expulsions against the bottom of the tube with bulb and pipette. Phase microscopy showed the organisms to be present as individuals or small groups, and plate counts on 25% blood agar showed there were about 4 x 10^5 organisms (or clumps) in the 0.05 ml of inoculum introduced into the tissue cultures. At appropriate intervals after inoculation, coverslip cultures were prepared for microscopic study by washing twice in BSS, fixation in 10% neutral formalin, staining with carbol-fuchsin, destaining with 1% HCl in 95% ethanol, and counterstaining for 10 minutes with Giemsa. The media were changed in the remaining cultures after 2 days. All incubations were at 37°C. Suspensions of carbon were prepared from India ink according to Hanks(4). The experimental media used were variations of medium A, that is, 50% BSS and 40% serum, usually human or horse. The sera were all inactivated at 56°C for 30 minutes. Ten per cent chick embryo extract was also present in the media used in the present work, but the described effects on phagocytosis will occur in its absence.

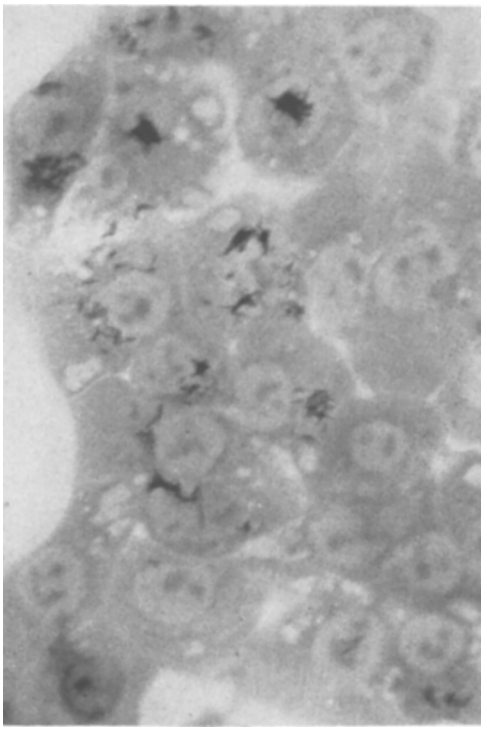

FIG. 1. HeLa cells, horse serum medium, one day after addition of tubercle bacilli. Many of the cells have taken bacilli into the cytoplasm.

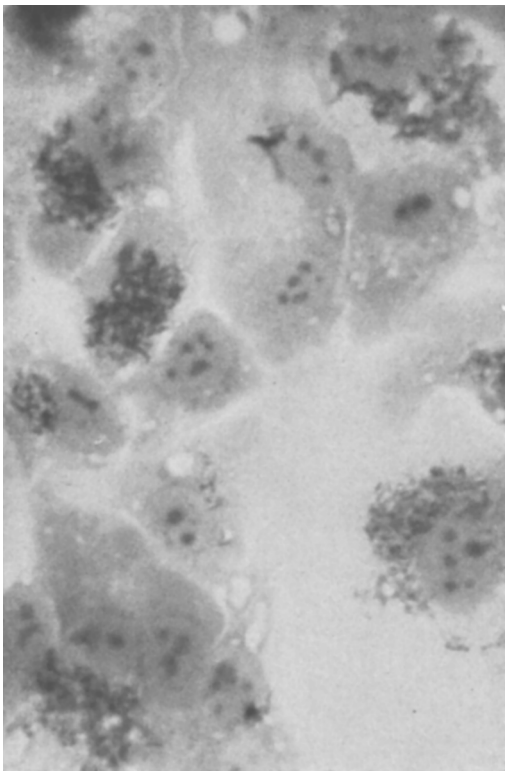

FIG. 2. HeLa cells, horse serum medium, 3 days after addition of tubercle bacilli. The bacilli in the cells have multiplied to fill much of the cytoplasm.

Results. Fig. 3 and 4 show the results obtained when the media contained human serum. Only a few cells (less than 1%) be-

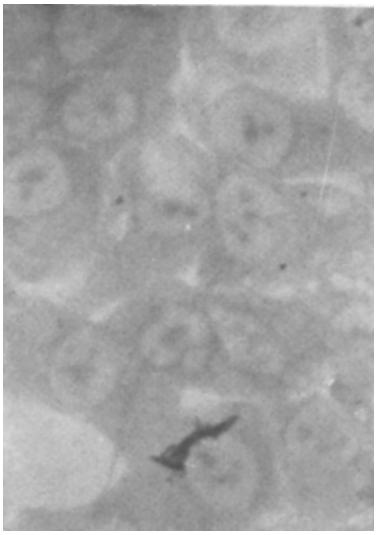

FIG. 3. HeLa cells, human serum medium, one day after addition of tubercle bacilli. Only an occasional cell has taken in bacilli.

came infected. However, when the media contained a satisfactory horse serum, 50% or more of the cells became infected (Fig. 1). Tubercle bacilli within the cells then grew rapidly so that within 3-5 days large masses of bacilli were seen in the cytoplasm(Fig. 2). Smears of the culture fluid, which were done routinely, showed that although growth occurred in the fluid with the stated inoculum, no significant difference could be noted between the horse and human serum media.

The intracellular location of the organisms as seen in Fig. 1 and 2 was evidenced by the following observations. The bacilli, after a few days, were located around the nucleus with special concentration at the cytocentrum, as is characteristic of cytoplasmic inclusions. Washing the cells with BSS and changing to fresh media of the same constitution each day did not decrease the amount of intracellular growth. The inclusion of streptomycin in the culture media in concentrations of 4 γ/ml did not affect intracellular growth, although this concentration was enough to prevent extracellular growth. A change of the medium to one containing human serum did not alter bacillary growth within the cells. Lastly, sections of infected cells cut perpendicularly to the plane of growth showed the bacilli to be surrounded by cytoplasm.

That this effect was due to increased phagocytosis rather than to an increased invasive property of the bacilli as a result of growth in the tissue culture medium is indicated by experiments with nonliving particles. If the inoculum was a suspension of heat-killed tubercle bacilli or carbon particles, or both, these particles were found within the HeLa cells in much greater numbers with horse serum media than with human serum media (Fig. 5 and 6).

A number of other culture media have been tried but none has given as much phagocytic activity as the horse serum media described. Sera from other species, monkeys, rabbits, cattle, guinea pigs, and chickens, as well as bovine amniotic fluid, were also tried as substitutions for the serum in medium A. Although some samples of chicken serum and bovine amniotic fluid showed better results than human serum, none was as good as favorable samples of horse serum. Ultrafiltrates of horse, cow, rabbit, and human serum and bovine amniotic fluid were intermediate in effect and indistinguishable from each other. Not all samples of horse serum were effective and, despite extensive trials, we have not been able to predict without preliminary trial whether a certain lot will be suitable. However, repeated tests on a given lot have given entirely

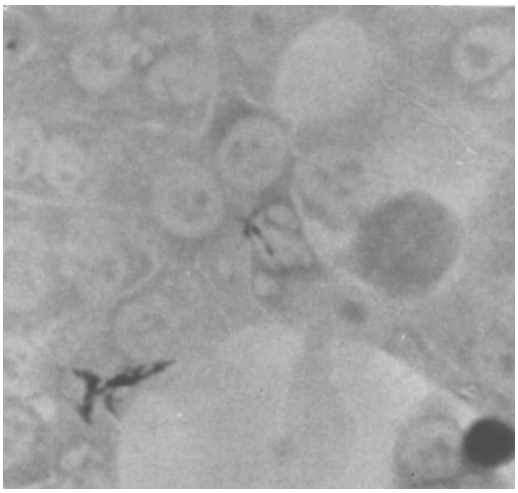

FIG. 4. HeLa cells, human serum medium, 3 days after addition of tubercle bacilli. Not much change from day 2 (Fig. 3), although it is possible to find cells containing considerable bacterial growth.

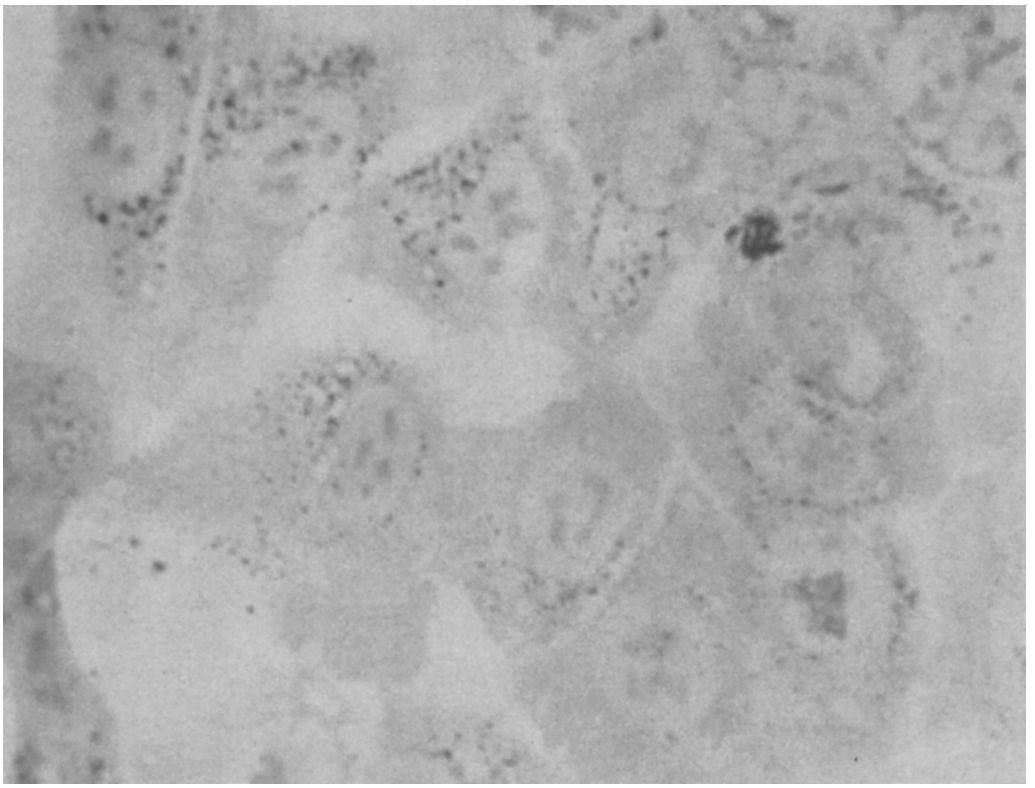

FIG. 5. HeLa cells, horse serum medium, one day after addition of carbon particles and dead tubercle bacilli. Many of the cells have taken the particles and bacilli into the cytoplasm.

uniform results over periods of at least 4-5 months.

Monkey kidney cell cultures in monolayers have shown essentially the same results, that is, favorable horse serum medium is able to increase phagocytosis of living and dead human tubercle bacilli and carbon particles. Luxuriant intracellular growth of the living tubercle bacilli then followed. The results in plasma clot cultures of human foreksin were not so easy to evaluate, perhaps because of the interposition of the clot between cells and culture medium.

The sensitivity of HeLa cell cultures in suitable horse serum culture fluid, as growth medium for small numbers of tubercle bacilli was indicated by several comparisons with bacteriological media, HeLa cell cultures detecting about as many bacilli (H37Rv) as Dubos medium with or without Tween (Difco), and about one-tenth as many as 25% human blood agar(5). It is interesting that the result in HeLa cells could be read in 3-5 days.

Because we were interested primarily in factors affecting intracellular bacillary growth (as a part of a study of tissue culture procedures which might be useful in a study of the leprosy bacillus), we have not attempted to develop methods for the accurate estimation of extracellular bacilli. The number of extracellular bacilli was studied only by smears of the culture fluid, and the strong tendency of the bacilli to clump subjects this method of estimation to considerable error. From the data obtained it is not possible to know whether the total amount of bacillary growth occurring in a culture tube, intra- and extracellularly, was increased as a result of the increased phagocytosis.

Discussion. Certain difficulties are met in interpreting results obtained in tissue culture according to the concepts of infectious processes which are derived mainly from the study of infections in intact animals. Thus it

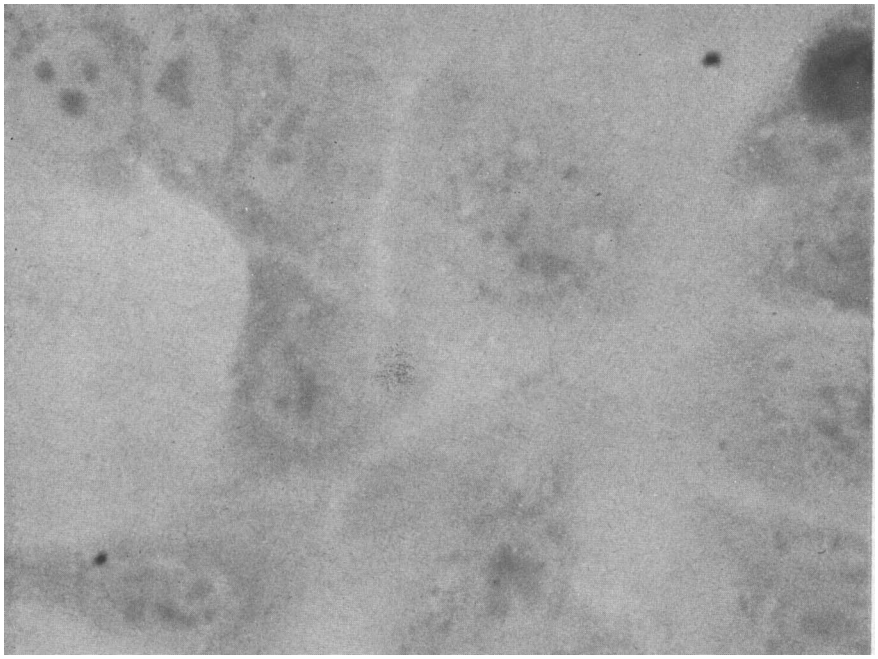

FIG. 6. HeLa cells, human serum medium, one day after addition of carbon particles and dead tubercle bacilli. The intake is considerably less than that in horse serum (Fig. 5), although in the original preparations some small carbon particles may be seen in the cytoplasm of many cells.

is said that a certain cell or tissue culture is susceptible or resistant to a particular infectious agent, because of the practice of considering a mouse or guinea pig as being susceptible or resistant to a given agent. Obviously a more complete statement of experimental conditions is desirable with tissue cultures because of the greater possibility of affecting the multiplication of the infectious agent by changes in culture media, temperature, pH, conditions of inoculation, etc. Even the term "susceptibility" tends to become ambiguous, because in this study, for example, it appears that HeLa cells in our medium are resistant to human tubercle bacilli, yet after the bacilli have gained access to the cytoplasm as a result of use of horse serum media they grow very well in HeLa cells in 40% human serum. A further understanding of the processes by which phagocytosis occurs in a cell such as the HeLa cell may emphasize other semantic obstacles.

Summary. When human tubercle bacilli are added to cultures of HeLa cells, the amount of intracellular growth that ensues depends upon the culture medium on the HeLa cells. If the medium is one containing 40% human serum, 10% chick embryo extract, and 50% BSS, little growth is seen within cells. However, if instead of human serum, certain horse serums are used, tubercle bacilli are rapidly taken up by the cells and luxuriant intracellular growth follows, even if the culture medium is later changed to one containing human serum in place of the horse serum. The mode of action of the horse serum media appears to be due to an increase in phagocytosis, since such media also increase the amount of phagocytosis of dead tubercle bacilli and carbon particles.

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