USE OF HEla CELLS INFECTED WITH TUBERCLE BACILLI FOR THE STUDY OF ANTI-TUBERCULOUS DRUGS

CHARLES C. SHEPARD


Received for publication October 8, 1956

It has been reported elsewhere that the growth of tubercle bacilli in HeLa cells in monolayer is rapid enough to be readily apparent in a few days (Shepard, 1955). The present paper describes a method for the employment of this convenient tissue culture system for the study of anti-tuberculous drugs.

Mackaness (1952), Mackaness and Smith (1952), and Suter (1952) have used rabbit and guinea pig macrophages in vitro to measure the activity of anti-tuberculous drugs and have pointed out that some of these drugs are not fully active against intracellular bacilli. Thus, the minimal inhibitory concentration of streptomycin in the macrophage system was 10 to 100 times more than it was in the usual test in bacteriological media, whereas the minimal inhibitory concentration of isoniazid, isonicotinic acid hydrazide (INH) was the same in the macrophage system as in bacteriological media. This difference between streptomycin and INH was thought to arise from a relative impermeability of the cell membrane to streptomycin, so that the drug had to be present in excessive concentration in the extracellular fluid in order to achieve an adequate level within the cell. In contrast, the cell membrane was thought to be freely permeable to INH so that it was present in the same concentration within the cell as in the surrounding fluid. This conception took origin in the work of Magoffin and Spink (1951) who showed that brucella organisms were able to escape the action of streptomycin in bacteriological media if they were contained in human leucocytes.

The factor of apparent permeability of the cell to the drug was taken into account in the experimental arrangement described below by the adoption of two alternative schedules for the administration of the drug. The two schedules were based on the observation that in this system the phagocytosis of tubercle bacilli is largely confined to the first day after the addition of the bacterial suspension to the tissue culture fluid. In the "early" schedule, drug treatment was initiated before infection, so that the drug was present during the period of phagocytosis and was maintained at the same level until the termination of the experiment. In the "delayed" schedule, the drug was withheld until the second day, at which time phagocytosis was largely complete, so that the bacilli had already reached their intracellular position before the drug was present in the tissue culture fluid.

MATERIALS AND METHODS

Most of the technique employed has been described elsewhere (Shepard, 1955). Infection of the cells was achieved by taking advantage of the marked increase in phagocytosis that occurs in media containing selected horse sera. Thus HeLa cells were grown on coverslips for one or two days in 40 per cent human serum, then they were washed with BSS, a tissue culture medium consisting of 40 per cent horse serum added, and a suspension of tubercle bacilli (strain H37Rv) was introduced in 0.05 ml of BSS. On the next day the cells on the coverslips were again washed in BSS, and a tissue culture medium consisting of 40 per cent human serum was added. Two days later, or three days after the addition of the tubercle bacilli, the coverslips were washed in BSS, fixed in 10 per cent neutral formalin, and stained. Microscopical examination with the 10 x or 40 x objective readily revealed the amount of intracellular growth.

The amount of medium per tube was one ml. Incubation was carried out at 37 C.

As has been stated, the drug was administered by one of two schedules. In the "early" schedule it

1 Presented in brief at the meeting of the Society of American Bacteriologists in Houston, May 1956.

2 Throughout the paper 40 per cent serum refers to 40 per cent serum plus 60 per cent balanced salt solution (BSS) (Hanks and Wallace, 1949).
was present in the tissue culture medium that consisted of 40 per cent horse serum as well as in the following 40 per cent human serum, and thus was present during and after phagocytosis. By the "delayed" schedule, however, it was not added to the 40 per cent horse serum, but was added to the subsequent 40 per cent human serum, so that it was present only after the bacilli had reached an intracellular position.

Drugs were routinely studied at five different concentrations on each occasion, thus requiring five tubes plus an untreated control for each schedule, or twelve tubes for both schedules. The concentrations chosen the first time included the minimal inhibitory concentrations in bacteriological media and were varied by a factor of four. The concentrations chosen for repeat determinations were varied by a factor of two, and the range covered depended upon the earlier findings. All the drugs were studied on at least two occasions on both schedules. Successive determinations of the minimal inhibitory concentration for a drug were always in agreement, except with INH where some variation was encountered with this labile drug. Also the amount of cytotoxicity observed at a given level of drug was not always the same in different tests.

Minimal inhibitory concentrations were also determined in liquid bacteriological media in 5 ml of "tween"-albumin media (Difco Tb-tween). For these determinations the inoculum was the same as that used for the HeLa cell cultures, and readings of turbidity were made after about one week at 37 C.

**EXPERIMENTAL RESULTS**

The results are given in table 1. The minimal inhibitory concentration stated was that found in two to four separate determinations. It will be noted that the drugs were divided into the two groups in the upper and lower half of the table. In the upper half are found those drugs which were fully effective against tubercle bacilli within the HeLa cells. For example, the values found for cycloserine were the same in HeLa cells by the "early" schedule and "delayed" schedule and also the same in bacteriological media in the absence of cells. INH and pyrazinamide gave the same type of result.

In contradistinction, the drugs in the lower half of the table did not exhibit full activity against intracellular bacteria, so that when administered by the "delayed" schedule a higher figure was found than by the "early" schedule, and the lowest figure of all was found in bacteriological media. Streptomycin exhibited this difference most strikingly. One μg/ml of this drug was enough to stop growth in the bacteriological media, whereas not even 2000 μg/ml in the tissue culture fluid affected the growth of bacilli within cells. (At higher con-

**TABLE 1**

*Minimal inhibitory concentrations of drugs against tubercle bacilli (strain H37Rv) growing in HeLa cells or in bacteriological media*

<table>
<thead>
<tr>
<th>Drug</th>
<th>Minimal Inhib. Conc. (μg/ml)</th>
<th>Values from Literature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In HeLa cells</td>
<td>In bacteriol.</td>
</tr>
<tr>
<td></td>
<td>Early schedule</td>
<td>Delayed schedule</td>
</tr>
<tr>
<td>Cycloserine</td>
<td>8-16</td>
<td>8-16</td>
</tr>
<tr>
<td>INH (isoniazid)</td>
<td>0.016</td>
<td>0.016-0.128</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>128-256</td>
<td>128-256</td>
</tr>
<tr>
<td>PAS</td>
<td>1-4</td>
<td>1-6 (64+)</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>4-16</td>
<td>&gt;2000</td>
</tr>
<tr>
<td>Tetracycin</td>
<td>1-16</td>
<td>16- (64+)</td>
</tr>
<tr>
<td>Viomycin</td>
<td>64-256</td>
<td>&gt;2000</td>
</tr>
</tbody>
</table>

For HeLa cells the lesser concentration refers to partial inhibition, the higher to complete inhibition except where the numbers are in parenthesis, in which case cytotoxicity was seen at the concentration indicated. By the early schedule the drug was present while the bacilli were entering the cells; by the delayed only after they had entered the cells. For bacteriological media complete inhibition only is referred to.

centrations HeLa cell morphology was altered.) When streptomycin was present during the period of phagocytosis ("early" schedule), amounts of 4 to 16 μg were enough to decrease the later growth of the tubercle bacilli within cells. Similar but less pronounced differences in the minimal inhibitory concentrations were observed with the other drugs of this group, PAS, tetracyclin, and viomycin.

With PAS and tetracyclin changes in cell morphology (primarily nonspecific rounding) occurred at concentrations at which the bacillary growth was only partially inhibited. For this reason the concentrations giving complete inhibition in apparently healthy cells could not be determined, and the figure in parenthesis in the table refers to the level at which cytotoxicity was observed.

**DISCUSSION**

The results confirm and extend the earlier findings of Mackaness (1952) and Suter (1952) that certain anti-tuberculous drugs, such as streptomycin, are not able to exert their full activity against intracellular bacilli, whereas others, such as INH, are fully active against bacilli within the cell. The present findings substantiate the earlier ones in the case of INH, PAS, streptomycin, and viomycin. Since Mackaness found oxytetracyclin to be fully effective within the cell, the current result with tetracyclin might not have been expected, and whether this difference is due to factors associated with the chemistry of the drugs or the properties of the cells is not now apparent.

The present work adds cycloserine and pyrazinamide to the drugs able to exert their full activity against intracellular tubercle bacilli. Although pyrazinamide is related to INH, which had earlier been shown to be active in animal macrophages, cycloserine is apparently unrelated. Thus although the action of INH and pyrazinamide is restricted largely to mycobacteria, cycloserine has a broad range of activity against many bacterial species. It is interesting to note that the molecular weights of the compounds in the upper half of the table are in the range of 100 to 200, and those in the lower half, with the exception of PAS, are larger than 400.

Mackaness (1952) and Suter (1952) followed the explanation of Magoffin and Spink (1951) in saying that streptomycin was not able to enter the cell. However, Eagle and Saz (1955) have pointed out that physiological differences might exist between the bacilli growing in bacteriological media and those within cells, and have suggested that streptomycin's relative ineffectiveness is due to the slowness of the growth of tubercle bacilli in macrophages. The present results with HeLa cells would not appear to confirm this suggestion because although the tubercle bacilli grow faster in HeLa cells than in macrophages, concentrations of streptomycin much higher than those found inhibitory to bacilli in macrophages were without effect in HeLa cultures.

Eagle and Saz (1955) state that experiments with radioactive streptomycin indicate that the antibiotic does get into certain mammalian cells. It would be interesting to explore the relative concentrations of radioactive samples of the drugs in table 1 in a cell such as the HeLa cell, which provides favorable growing conditions to tubercle bacilli and also possesses an apparent impermeability to certain of the drugs. The behavior of the HeLa cell toward streptomycin may be influenced by its previous growth in media containing the antibiotic.

In considering the factor of impermeability it would seem well to remember that cells such as HeLa cells and macrophages are constantly engaged in the taking in of particles (phagocytosis) and also of droplets (pinocytosis) (Gey et al. 1954). After the droplets are ingested they move quickly toward the cytocentrum, usually decreasing rapidly in size as they move. Consequently if such a cell were able to maintain a concentration difference of a substance across the cell membrane it would apparently need to be capable of excreting the substance selectively. It is possible that all materials in the tissue culture fluid would be present within what is ordinarily conceived of as the cell boundary, although in the case of certain substances this might represent only a temporary stay while awaiting deportation.

The ability of a chemotherapeutic agent to exert its activity against organisms located inside cells is an important point in the case of mycobacterial infections. In tuberculosis an intracellular phase is thought to occur early in the infection. Later in the disease most of the organisms are found outside of cells, and the ability of a drug to operate against intracellular organisms does not seem essential. In leprosy, however, the bacilli at all stages are located primarily within cells and for a drug to be fully effective it would presumably need to act on intracellular organisms. Since the etiologic agent of leprosy has not been cultivated,
nor has the disease been transferred to laboratory animals, drugs cannot be evaluated for their activity against *Mycobacterium leprae* except by therapeutic trial in patients with leprosy. Trials in humans are time-consuming and difficult in a disease as chronic and variable as leprosy, so that not many drugs can be adequately tested. The usual basis for selection of drugs for such trial has been the activity of the drug against *M. tuberculosis*, and it would seem worthwhile to give special consideration to those drugs that are active against the intracellular organisms. In this connection efforts have been made to assess the sulfone drugs in the system herein described, but the low solubility of DDS, the parent compound, in the tissue culture fluid has interfered with the evaluation. This aspect continues under investigation.

The concentration of pyrazinamide found to be active against intracellular bacilli may give some indication of intracellular pH in the HeLa cell, at least in the immediate environment of the tubercle bacilli, since McDermott *et al.* (1954) have shown that the minimal inhibitory concentration of this drug depended on the pH of the bacteriological medium. On this basis an intracellular pH of about 6.0 would be indicated.

In discussion of the HeLa cell system it may be useful to mention some of its apparent advantages and disadvantages in comparison to the in *vivo* methods in use, which are principally the treatment of experimentally infected mice and guinea pigs. The amount of drug required for the tests in HeLa cells is of the order of that needed for determinations in bacteriological media, and thus less than that needed for the treatment of animals. The result could be learned more quickly than by the other in *vivo* methods. There would appear to be certain inherent advantages in the use of human type tissue for the study of drugs to be used in the treatment of human disease. It is now well known that human cell lines, such as HeLa cells, strongly reflect the susceptibility and resistance of human beings to virus diseases. The same relationship seems to hold for tubercle bacilli and other mycobacteria, and virulent human and bovine tubercle bacilli grow much more rapidly than strains of lesser virulence such as BCG and H37Ra (Shepard, 1955a). That there may be critical differences between human tuberculosis and the experimental infection in guinea pigs and mice is indicated by the failure of cycloserine to affect the disease in these animals, whereas it is fairly effective in the human disease (Steenken and Wolinsky, 1956).

On the other hand the tissue culture method does not allow the investigation of the factors observable only in intact animals, for example, the role played by absorption and excretion, and the possible conjugation or inactivation of the drug in a different manner by the intact animal than by the HeLa cells and tissue culture fluid. Furthermore, although toxicity by the drugs was observed as morphological changes in the HeLa cells, it is not known whether these are reflections of the toxicity that might be observed in the human being.

**ACKNOWLEDGMENT**

The author wishes to express his appreciation for the capable technical assistance of Mrs. Mary E. Jones and Miss M. Nannett Green. Samples of cycloserine were made available by Dr. Michael G. Mulinos of Commercial Solvents Corporation, and Dr. Raymond M. Rice of Lilly Research Laboratories; and pyrazinamide by Dr. Nelson H. Schimmel of Sharp and Dohme.

**SUMMARY**

A system is described for the study of the activity of drugs against tubercle bacilli growing in cells of human origin (HeLa cells).

The effective drugs can be divided into two groups. In the first group were the drugs with full activity against bacilli already within the cells. Thus the same minimal inhibitory concentrations were observed with an "early" schedule of drug administration, in which the drug was present during the period of phagocytosis, as by a "delayed" schedule, in which the drug was withheld until the bacilli had entered the cell. In addition, the same minimal inhibitory concentration was found in the usual tests in bacteriological media. This group of drugs was made up of cycloserine, isonicotinic acid hydrazide (isoniazid), and pyrazinamide.

In the second group were found those drugs exhibiting decreased activity against intracellular bacilli, so that higher minimal inhibitory concentrations were found by the "delayed" than by the "early" schedule in HeLa cells, and both of these values were higher than those found in the absence of cells in bacteriological media. Into this second group of drugs fell PAS, streptomycin, tetracyclin, and viomycin.
REFERENCES


