CELL-MYCOBACTERIA-INFECTION SYSTEM

I. STUDIES OF THE EFFECTS OF VARIOUS KINDS OF SERUM ON THE PHAGOCYTOSIS OF MYCOBACTERIA BY L CELL

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Shepard¹⁾⁻³⁾ has been making many contributions to the study of host-parasite relationships by researching the behaviours of parasites and host cells in the course of bacteria infection, especially acid-fast bacilli to the established HeLa cells. Mori⁴⁾ presented results similar to that which were described by Shepard. Mitsuhashi et al.⁵⁾⁶⁾ reported on the conditions influencing the infections of *Salmonella enteritidis* to L cells which were derived from mouse fibroblast and the morphological changes of cells and bacteria after infection.

The purpose of this present paper is to describe the fundamental data in regard to the factors affecting the phagocytosis rate in the case of infection of acid-fast bacilli to the L cells.

MATERIALS AND METHODS

1. *The established cells :*

HeLa cell cultivation: The HeLa cells used in these experiments were obtained from the Division of Virology, Department of Microbiology of this University. Growth medium used was 0.5 % lactalbumin-Hanks solution (Melnik-Hanks [M+H]) containing 10 % inactivated bovine serum and antibiotics.

L cell cultivation: L cells used were grown in the growth medium containing : 0.1 % yeast extract, 0.5 % lactalbumin hydrolysate and 5 % inactivated bovine serum in Hanks (YLH) medium.

2. *Bacterial strains used :*

The following strains of *Mycobacteria* were used; *Myc. smegmatis*, BCG, H37Ra and H37Rv cultivated at 37°C for 2 weeks on Ogawa's medium, and H37Rv INH (isonicotinic hydrosate) resistant strains cultivated for 2 weeks on Ogawa's medium containing 100 µg of INH and, and H37Rv SM (streptomycin) resistant strain cultivated on Ogawa's medium containing 100 µg of SM. A Bacterial suspension was prepared in a physiological saline after grinding in the sterile agate-mortar and the supernate of centrifugation at 1500 r.p.m. for 5 minutes was used.

This suspension contained approximately 10^6 of bacterial cells per ml.

3. Serum employed :

The following kinds of sera were used in tissue culture media; Cattle, horse, goat, sheep, guinea pig and monkey, some of these sera were inactivated at 56°C for 30 min., while others were filtrated through a seitz E.K. filter in order to remove bacterial contamination.

4. General procedure :

The established cells were grown in the tubes at 37°C for 2 days in the growth medium which contained 5 % bovine serum, but no antibiotics. The medium was discarded after 48 hours' cultivation. The cells were then washed twice with phosphate buffered saline, and 1 ml of infection medium containing a half volume of a different serum was added. Thereafter, 0.1 ml of bacterial suspension was inoculated into each tube. After incubation at 37°C for 24 hours, the cells were washed three times with the physiological saline in order to possibly remove the extracellular bacteria and were stripped off from the tubes by rubber sticks. Then, the cells were suspended in 0.5 ml of the physiological saline and were smeared on slide glasses, dried, fixed with 10 % formalin solution for 1 or 2 days, and were stained with Ziehl's solution at 37°C for 2 hours and counter stained with Löffler's solution after decolourization with 3 % HCl alcohol. Two hundred cells were counted at random in representative areas. The rate of phagocytosis (percentage) was scored as follows :

$$\frac{\text{Numbers of cells acquiring bacilli}}{200 (\text{Numbers of cells countered})} \times 100$$

EXPERIMENTAL

1. Influences of sera used on phagocytosis of Mycobacteria by HeLa and L cells :

Shepard¹⁾ stated that the uptake of Mycobacteria by HeLa cells was highly promoted when an appropriate horse serum was used in the tissue culture medium as compared with other kinds of serum. In the present study, phagocytosis of H37Rv by HeLa cells was compared to that of L cells by using the same lot of various kinds of serum.

The results obtained are shown in Table 1.

The kind of serum which was most effective in the promoting of phagocytosis of H37Rv by HeLa cells was bovine serum; next; rat, horse and monkey serum. Phagocytosis when used with other sera was half of the phagocytosis rate of the above sera. However, the effect of bovine serum on phagocytosis obtained in this experiment was not as significant as reported by Shepard. On the other hand, in the case of L cells, the most effective serum for promoting phagocytosis was a guinea pig one, and a rabbit serum followed. Other sera used here had no effect on phagocytosis. Therefore, the behavior of HeLa cells was quite different from that of L cells even though the same lot of serum was used.

In order to reconfirm the effects of horse sera on phagocytosis, four lots of horse serum were used for HeLa cells and L cells. The results obtained in HeLa

TABLE 1

Effects of various kinds of serum on the phagocytosis of H37Rv strain by HeLa cells and L cells

Serum	HeLa cells	L cells
Horse	22%	21%
Bovine	25	10
Goat	13	17
Sheep	15	5
Rabbit	13	30
Guinea pig	11	36
Mouse	14	20
Rat	23	17
Monkey	20	18

cells as well as L cells demonstrated that there were slight differences of the effect among them, but these differences were not as remarkable as the results presented by Shepard.

In general, the phagocytosis rate of L cells was lower than that of HeLa cells. From the results described in Table 1, it was not due to the lot of the sera used that the phagocytosis rate of HeLa cells was not as high as the results obtained by Shepard.

TABLE 2

Effects of four lots of horse serum on the phagocytosis of BCG strain by HeLa cells and L cells

Lot	HeLa cells	L cells
1	34%	24%
2	29	23
3	25	21
4	26	20

2. *The kinds of serum influencing phagocytosis of Myc. tuberculosis hominis by L cells.*

The kinds of serum influencing phagocytosis of Mycobacteria by L cells was examined by using H37Rv, H37Rv SM resistant, H37Ra, and H37Rv INH resistant strains. The results are shown in Table 3. In this Table, conveniently, the phagocytosis rate of horse serum was presented as 1.0 and the effects of other sera were indicated as comparing to this rate. In the case of H37Rv and SM resistant H37Rv strain, rabbit and guinea pig serum promoted the phagocytosis by L cells. Phagocytosis of H37Rv INH resistant by L cells was promoted by the

use of guinea pig and monkey serum, but there were no sera which promoted phagocytosis of H37Ra. From these results, it could be noted that H37Rv was more readily acquired than H37Ra by the L cells, and that H37Rv INH and SM resistant strains were more easily acquired than its original strain, and that especially guinea pig and rabbit serum promoted the phagocytosis of those strains by the L cells.

TABLE 3

Effects of various kinds of serum on the phagocytosis of Myc. tuberculosis hominis by L cell

Serum	H37Rv	H37RvSM-r	H37RvINH-r	H37Ra
Horse	1.0	1.0	1.0	1.0
Bovine	0.6	1.2	1.4	0.8
Goat	0.5	1.3	1.2	0.8
Rabbit	1.3	2.5	1.4	1.1
Guinea pig	1.4	1.8	1.8	1.0
Mouse	0.6	1.5	1.4	1.1
Monkey	0.6	1.4	1.6	1.2
Phagocytosis rate of horse serum	37%	13%	36%	20%

In order to see if the difference of phagocytosis rates mentioned above depended upon the bacterial activities or the behaviour of the cell, formaline killed Mycobacteria were used in this experiment. In this test, H37Rv was more readily phagocytosed than H37Ra by L cells, the same as in the case of living Mycobacteria. In H37Rv strains, an original and INH resistant strain were more easily phagocytosed than other strains, and guinea pig, rabbit and monkey serum promoted the phago-

TABLE 4

Effects of various kinds of serum on the phagocytosis of formalin-killed Myc. tuberculosis hominis by L cell

Serum	H37Rv	H37RvSM-r	H37RvINH-r	H37Ra
Horse	1.0	1.0	1.0	1.0
Bovine	1.8	0.6	1.0	0.4
Goat	1.4	1.3	1.7	1.1
Rabbit	1.2	1.3	2.2	0.4
Guinea pig	2.2	1.5	2.7	0.9
Mouse	0.8	1.2	1.2	0.5
Monkey	2.2	1.0	2.7	0.3
Phagocytosis rate of horse serum	5 %	12%	6 %	15%

cytosis by the L cells. With only these results it would be difficult to determine that the differences of the phagocytosis rate would be present in the bacterial side or cell side.

3. *Comparison of the phagocytosis of H37Rv to that of Myc. smegmatis by L cells.*

The purpose of this experiment is to discover whether there are any differences in the phagocytosis between a virulent strain and an avirulent strain by the L cells. H37Rv strain was used as a virulent strain and Myc. smegmatis as an avirulent one. The results obtained are shown in Table 5, in which it is demonstrated that the avirulent strain was more readily phagocytosed than the virulent strain. The highest phagocytosis rate was demonstrated in the case of guinea pig serum as described above, but Myc. smegmatis was considerably phagocytosed by the L cells in the use of bovine and goat serum.

TABLE 5

Effects of various kinds of serum on the phagocytosis of Myc. tuberculosis hominis and Myc. smegmatis by L cell

Serum	H37Rv	Smegma
Horse	1.0	1.0
Bovine	0.7	13.7
Goat	1.9	13.0
Sheep	1.1	5.7
Rabbit	5.4	9.7
Guinea pig	9.0	21.3
Mouse	1.1	4.7
Phagocytosis rate of horse serum	7 %	3 %

4. *The effect of guinea pig serum on the phagocytosis of Mycobacteria by the L cells.*

The results obtained above indicate that, in general, guinea pig serum promoted the phagocytosis rate by the L cells. The fresh guinea pig serum was added to the inactivated guinea pig serum at various rates for the purpose of studying the promoting factors of the guinea pig serum. Table 6 indicates the fresh guinea pig serum promoted the phagocytosis rate when the volumes of fresh serum added were increased. When the fresh guinea pig serum was inactivated by heating at 56°C for 30 minutes, its promoting effect of the phagocytosis was lost.

DISCUSSION

Shepard¹⁾ has been reporting on the study of phagocytosis of bacteria, especially Mycobacteria by HeLa cells which was derived from human tissue. He de-

TABLE 6

*Effects of fresh guinea pig serum on the phagocytosis of BCG strain by L cells **

Inactivated bovine serum	Fresh guinea pig serum	Phagocytosis rate (%)
100%	0 %	34
90	10	34
80	20	40
70	30	44
60	40	44
0	100	61

* Serum was added to the medium at a concentration of 2.5%.

TABLE 7

Effects of inactivated guinea pig serum on the phagocytosis of BCG strain by L cells

Serum	Phagocytosis rate (%)
Inactivated bovine serum	34
Fresh guinea pig serum	61
Inactivated guinea pig serum	31

monstrated that the HeLa cells easily acquired Mycobacteria when an appropriate horse serum was used in the tissue culture medium. That is, the phagocytosis rate in this system was more than 50 %, whereas the phagocytosis rate of HeLa cells was less than 1 % when human serum was used, and that bovine fetal serum was found to be more effective than horse serum. The authors employed the L cells which were derived from mouse fibroblast in order to study the cell-Mycobacteria-infection system. The reason why the L cells were employed in this experiment was that the L cell-Mycobacteria-infection system would be utilized for a part of the studies on the experimental tuberculosis in mouse and for an attempt of the fundamental experiment on tissue cultures of *Myc. leprae murium* which could be reported in the near future. However, it would be very doubtful whether these attempts would be suitable for the purpose of simplifying the studies on host-parasite relationships, because Mycobacteria, (except *Myc. lepra* and *lepraemurium*), are not obligate parasites such as viruses and rickettsia, and also the activities of the L cells would differ from the monocyte which plays an important role in experimental animal tuberculosis, but it would be presumed that the L cells might contain some genetic factors of the mouse.

From the results obtained here it is clear that the L cells acquired acid fast bacteria and that some kinds of serum in the culture medium influenced the course of phagocytosis of acid fast bacteria as was observed by Shepard in the case of

HeLa cells. Although it would be conceivable that this promoting effect in the serum for phagocytosis might be due to the complement-like substance because this effect was inactivated by heating at 56°C for 30 min., this promoting factor should be precisely studied in the future in connection with opsonin and other agents.

There are some problems concerning the relationship between the number of cells and the number of bacterial cells pointed out by Bloch⁹⁾. In other words, it would be very difficult for a relationship between the number of cells and the number of bacterial cells to be maintained at a definite rate. Therefore, the phagocytosis rate in each tissue culture experiment could be changed by this relationship. For example, the effect of serum was very slight when a great number of bacteria was used. On the contrary, when a very few bacterial cells were used, the effect of the serum was increased.

In spite of the presence of many obstacles and opposing factors in this experimental procedure, it might be thought that the tissue culture system of L cells-Mycobacteria would be a recommended method when the bacterial infection to the cell could be separately observed in each phase in order to approach the in vivo system rather than the in vitro one.

SUMMARY

The effects of sera on the infection of Mycobacteria to the established cell derived from mouse were investigated in vitro. The cells used were L cells which were derived from mouse fibroblast and HeLa cells, and the strains of Mycobacteria employed were Myc. tuberculosis hominis H37Rv, H37Rv-INH-resistant, SM-resistant, Myc. tuberculosis bovis BCG and saprophytic Mycobacteria smegmatis, respectively. The following results were obtained.

1. There were some sera having different effects in the course of phagocytosis of Mycobacteria by the L cells and HeLa cells.
2. In general, it was demonstrated that the guinea pig serum promoted phagocytosis of Mycobacteria by the L cells. This promoting effect of the serum might be due to the complement-like substance because the effect was inactivated by heating at 56°C for 30 min.

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