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Quantitative aspects of *Mycoplasma pneumoniae*-cell relationships in cultures of lung diploid fibroblasts

By N. M. LARIN, N. V. SAXBY and D. BUGGEY
Therapeutics Research Division, Pfizer Ltd, Sandwich, Kent

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INTRODUCTION

This report describes quantitative studies on the growth of *Mycoplasma pneumoniae* in cultures of the WI–38 diploid cell strain. Several aspects of this were investigated, and as a result an interesting model of host-parasite relationship emerged. The feasibility of extending this model to the quantitative assay of antibiotics is discussed. According to recent observations the inhibitory effect of antibiotics may be strongly affected by the milieu in which mycoplasmas are perhaps protected by their close association with cells (Gori & Lee, 1964; Friend, Patuleia & Nelson, 1966). That cell attachment involves an active biological process on behalf of either cell or mycoplasma has recently been reported by Fogh & Fogh (1967), who have also convincingly demonstrated that quantitative models of mycoplasma-cell interactions may lend themselves to more meaningful studies of effects of antibiotics.

The significance of the capacity of some mycoplasmas to reside in the cytoplasm was first recognized by Hayflick & Stinebring (1955). This provided impetus for further investigations of mycoplasmas in cell culture (Hayflick, Stinebring, Breckenridge & Pomerat, 1956; Hayflick, 1956; Hayflick & Stinebring, 1960). Morphological studies of *M. pneumoniae* in experimentally infected monkey kidney cells were made by Clyde (1961).

Subsequently, mycoplasmas were frequently found as contaminants in a variety of cell cultures. The possible source of this type of contamination, the problems involved and the means by which cell cultures could be decontaminated were reviewed by Hayflick (1965*b*), Hayflick & Chanock (1965) and MacPherson (1966).

The experimental results to be described below are each based upon six separate assay dates.

MATERIALS AND METHODS

Mycoplasma

The FH strain of *M. pneumoniae*, kindly provided by Dr L. Hayflick, was used in these studies. This strain had undergone 156 consecutive passages in acellular media. Before these experiments, it had not been passaged in cell cultures.

Cell cultures

Cell monolayers of the WI–38 diploid cell strain were prepared in 4 in $\times \frac{1}{2}$ in tubes by the standard methods described by Hayflick (1965*a*).

Acellular media

The media used (PPLO broth and agar) were those described by Chanock, Hayflick & Barile (1962). These media contained beef heart infusion base (Difco) and fresh yeast extract (Hers) as described by Lemcke (1965).

Inoculation of cell cultures

Inocula. These were prepared by diluting with SM 199 a 3-day-old mycoplasma culture in the liquid acellular medium. The SM 199 contained no serum in order to avoid inhibitors. Serial tenfold dilutions of the inocula thus prepared were seeded on agar plates to determine retrospectively the number of viable organisms in the inocula.

Procedures. The growth medium was removed from confluent cell monolayers and replaced with 1 ml. mycoplasma inoculum. Inoculated cell cultures were incubated at 36°C. for periods up to 6 days.

Multiplicity of infection

In these quantitative studies it was important to be able to define the multiplicity of infection (i.e. the number of mycoplasmas in the inoculum per host cell). The average number of cells of the WI–38 strain grown as confluent monolayers in tubes was calculated to be approximately 5×10^5 by a method described by Larin & Roberts (1963). From this figure, the multiplicity of infection was calculated for each mycoplasma inoculum.

Mycoplasma recovery and enumeration from cell cultures and from broth

The number of viable mycoplasmas, cell-associated or extra-cellular, was determined for each incubation day. The supernatant fluid from four inoculated cell monolayers was pooled and the cells were washed three times using 1 ml. of SM 199 per tube for each wash. The washed cell monolayers were removed with a rubber scraper and the cells were resuspended in 4 ml. of fresh SM 199, in which they were disintegrated using an MSE homogenizer to liberate the cell-associated mycoplasmas.

Serial decimal dilutions of the cell culture supernatant and the cell homogenate were made separately in SM 199. Similar dilutions of PPLO broth cultures were made in PPLO broth. Viable counts were made by the method of Miles & Misra (1938), modified by using one drop of 0·05 ml. per plate, which was spread over the surface of the agar with a platinum loop. If the count was expected to be low, duplicate plates were each inoculated with three drops of 0·02 ml., which were not spread with a loop but left to form circular patches of about 20 mm. diameter. All plates were incubated for 10 days at 36°C, in closed chambers to prevent further drying of the medium.

Colonies were counted at a magnification of × 25, using a Zoom stereoscopic microscope (W. Watson & Sons, Ltd, London).

The reliability of the colony counting method was examined for conformity with a Poisson distribution and was found to be satisfactory. The colony counts of

consecutive decimal dilutions of mycoplasma cultures were compared and found to be very near to the expected decimal relationship.

Microscopical demonstration of mycoplasma effects on cell cultures

Cultures of infected cells on coverslips were washed three times in phosphate buffered saline, pH 7·3, to remove extracellular mycoplasmas, and then the cells were stained with orcein by a similar method to that of Fogh & Fogh (1964). The coverslips were examined under the microscope at magnifications from $\times 400$ to $\times 3200$.

Wherever other materials or techniques have been used, they are described in the text below.

RESULTS

The survival of mycoplasmas in SM 199 *and in SM* 199 + *WI–38 cell extract*

A mycoplasma inoculum sufficient to give an initial colony count of ca. 10^4 viable particles/ml. in liquid media was thought desirable for this study in order to allow conditions of maximal survival. For this purpose a mycoplasma culture in PPLO broth which contained ca. 10^6 viable particles/ml. was diluted 1/100 in PPLO broth, SM 199 or SM 199 + WI–38 cell extract and these media were incubated at 36° for 7 days. As shown in Table 1, the survival of *M. pneumoniae* in SM 199 alone or in SM 199 + WI–38 cell extract was very poor.

Table 1. *Growth of* Mycoplasma pneumoniae *in PPLO broth, and in SM 199 with and without WI–38 cell extract*

	Viable count (CFU/ml.) in		
Day	PPLO broth	SM 199	SM 199 with WI–38 cell extract
2	81,100	120 (0·86)	80 (0·57)
4	5,760,000	0	0
7	1,455,000	0	0

Inoculum, $1{\cdot}4 \times 10^4$ CFU/ml. The figures in parentheses are percentages of the original inoculum

With smaller inocula no mycoplasmas were recovered from SM 199 or SM 199 + WI–38 cell extract 24 hr. post inoculation. As a result of these experiments it is concluded that with massive inocula as above a small number of organisms may survive for 48 hr. in SM 199 due to materials carried over from the PPLO broth.

WI–38 cell-associated mycoplasmas in relation to extracellular mycoplasmas and to the multiplicity of infection

As shown in Table 2 and Fig. 1, the amount and the time of appearance of cell-associated and extracellular mycoplasmas were related to the number of CFU/ml. in the inoculum and thus to the multiplicity of cell infection. With a relatively high multiplicity, such as 0·095 ($10^{4{\cdot}7}$ CFU/ml. inoculum), no apparent lag phase occurred, although this could not be excluded, and the amounts of cell-associated and extracellular mycoplasmas were similar on each day of the 6-day experimental period.

At lower multiplicity of infection, there followed a definite lag phase when no mycoplasmas, cell-associated or extracellular, were detectable. Following the lag phase, the cell-associated mycoplasmas were always the first to appear and the extracellular mycoplasmas could not be detected till 1 day later. The lag phase became longer as the multiplicity of infection decreased (Table 2). The growth from the zero point after the lag phase was extremely rapid with the average generation time of 2·72 hr. (cell-associated: 2·71–2·79; extracellular: 2·65–2·75). There was also a linear relationship between increases of the cell-associated and extracellular mycoplasmas (Fig. 1). This finding is what could be expected on the hypothesis of the extracellular mycoplasmas being derived from cell-associated replication.

Table 2. *The amount and time of appearance of cell-associated and extracellular mycoplasmas in infected cell cultures*

	Multiplicity of infection							
	0·095		0·0079		0·00052		0·00007	
	Inoculum concentration*							
	4·7 (47·600) Mycoplasma count*		3·6 (3·930) Mycoplasma count*		2·4 (260) Mycoplasma count*		1·5 (35) Mycoplasma count*	
Days after inoculation	Cell-associated	Extracellular	Cell-associated	Extracellular	Cell-associated	Extracellular	Cell-asosciated	Extracellular
1	5·0 (99,900)	4·9 (88,700)	0	0	0	0	0	0
2	5·9 (856,000)	5·7 (529,000)	0	0	0	0	0	0
3	6·7 (4,860,000)	6·4 (2,720,000)	2·4 (260)	0	0	0	0	0
4	6·8 (5,960,000)	6·7 (5,160,000)	5·3 (221,000)	4·1 (12,600)	1·9 (80)	0	0	0
5	6·5 (3,160,000)	6·6 (4,300,000)	6·3 (2,060,000)	5·5 (286,000)	5·2 (150,600)	4·0 (9,900)	0	0
6	6·2 (1,596,000)	6·3 (1,956,000)	6·7 (4,880,000)	6·5 (2,860,000)	6·3 (1,806,000)	5·3 (202,000)	1·0 (10)	0

* $\log_{10}$ CFU/ml. with numbers of CFU/ml. in parentheses.

In summary, the data in Table 2 and Fig. 1 appear to fit a conclusion that cell infection of a relatively high multiplicity, such as 0·095, results in saturation of the cell monolayer, most likely explained by rate of cell attachment, with excess mycoplasmas passing freely to the supernatant fluid. At lower multiplicity, all mycoplasmas in the inoculum attach to the cells and there follows a lag period of 2 to 5 days during which infection is cell-associated. The occult period, during which the micro-organism is not recoverable either from the supernatant or from the cells, is a phenomenon of some considerable theoretical and practical interest.

Table 2 and Fig. 1 show that, though mycoplasmas were recoverable from an inoculum containing as little as $10^{1·5}$ CFU/ml., none of a 100 times greater amount of mycoplasmas, presumably present in a homogenate of cells infected with the $10^{3·6}$ CFU/ml. inoculum, could be recovered from it on agar plates during the first

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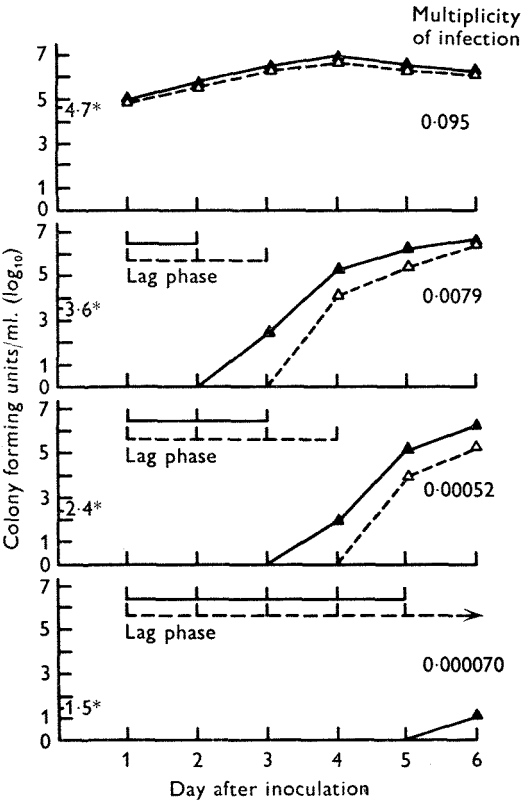

Fig. 1. The amount and time of appearance of cell-associated and extracellular mycoplasma in infected cell cultures. ▲—▲, Cell associated; △- -△, extracellular. * inoculum concentration.

Table 3. *Average yields of mycoplasmas (Log_{10} CFU/ml.) from cell culture and acellular medium*

Time after inoculation		Cell culture*			
Days	Hours	Cell-associated	Extra-cellular	Total	Acellular medium†
1	8	—	—	—	4·62
	24	5·18	5·23	5·50	5·00
	32	—	—	—	5·58
2	48	6·40	6·38	6·69	5·90
	56	—	—	—	6·11
3	72	6·64	6·61	6·92	6·27
	78	—	—	—	6·25
4	—	6·57	6·60	6·88	‡
5	—	6·38	6·39	6·68	—

* Inoculum concentration = $10^{4·52}$ CFU/ml., multiplicity of infection = 0·07
† Inoculum concentration = $10^{4·55}$ CFU/ml.
‡ Experiment discontinued.

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2 days after cell infection, and on day 3 the cells only yielded approximately 7% of the mycoplasma content of the inoculum. After day 3, the amount of mycoplasmas in cell homogenates increased logarithmically, and they appeared also in the supernatant fluid.

Generation time in cell culture and acellular medium

The results of our experiments to compare the growth of *M. pneumoniae* in cell culture and in acellular medium which was inoculated with similar amounts of mycoplasmas ($10^{4\cdot52}$–$10^{4\cdot55}$) are shown in Table 3 and Fig. 2. The growth curve in

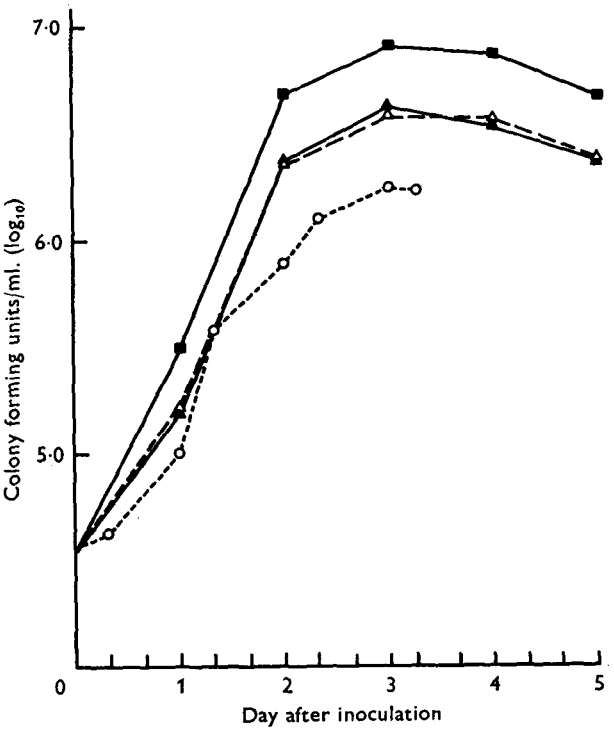

Fig. 2. Average yields of mycoplasma (Log_{10} CFU/ml.) from cell cultures and acellular medium. ▲—▲, cell-associated; △ – – △, extracellular; ■—■, cell-associated and extracellular; ○ – – ○, in acellular medium.

both cultures followed a similar pattern but a higher yield of the micro-organism was obtained in the cell culture. This shows that mycoplasma may prefer the cellular environment rather than acellular medium for their propagation.

Generation times during the logarithmic phase of growth were calculated and found to be as follows. For cell-associated and extracellular mycoplasmas, i.e. for the organism recovered from either the cell homogenate or the supernatant, the generation time was very similar, i.e. 6·1 hr. for each, and for the total from the cell homogenate and the supernatant fluid it was 6·0 hr. In ten similar experiments with inocula ranging from 4·36 to 5·21 $\log_{10}$ CFU/ml., the average generation time was

6·5 hr. for the cell-associated mycoplasmas and 6·2 hr. for the total of cell-associated and extracellular mycoplasmas (Table 4).

With smaller inocula, i.e. $10^{3\cdot6}$ to $10^{1\cdot5}$, which produced in infected cell cultures a lag phase during which no mycoplasma could be isolated for a period of 2 to 5 days (Table 2 and Fig. 1), the subsequent multiplication was extremely rapid with the average generation time of 2·72 hr. In contrast, the generation time in the acellular medium was 8·0 hr. Low & Eaton (1965) obtained average doubling times of 5 to 12 hr. in acellular medium. They found that the results obtained were influenced by the number of passages the organism had experienced. The FH strain of *M. pneumoniae* which was used in our experiments had undergone 156 passages in acellular media, but it had not previously been grown in cell culture. Therefore, the shorter generation time with this strain when grown in cell culture suggests more favourable conditions for mycoplasma propagation in association with the mammalian cell.

Table 4. *Average generation times for M. pneumoniae in cell culture*

Expt. no.	Inoculum concentration ($\log_{10}$CFU/ml.)	Multiplicity of infection	Mycoplasma generation time (h)	
			Cell-associated	Total cell-associated and extracellular
1	4·78	0·05–0·18	7·20	6·87
2	5·21		7·03	6·15
3	4·74		6·53	6·00
4	4·96		4·70	4·20
5	4·77		6·20	6·20
6	4·45		6·68	7·05
7	4·93		7·05	7·05
8	4·95		7·10	7·16
9	4·60		6·31	6·31
10	4·36		6·48	5·00
Average generation time			6·53	6·20

Effects of pH changes

Over the 6-day period of the experimental observations the pH values of the supernatant fluid in infected cell cultures was falling steadily from 7·1 or 7·2 to between 6·3–6·7 by the sixth day. The fall of the pH was greater in cell cultures infected with concentrated inocula, i.e. of the order of $10^{4\cdot5}$/ml. to $10^{5\cdot1}$/ml. mycoplasmas, but it was also observed when smaller inocula were used.

It was reported that with *M. pneumoniae* in acellular media there was a rapid loss of viability of the micro-organism once the pH value fell below 6·5 (Low & Eaton, 1965) and that no growth occurred at pH 6·0 (Shepard & Lunceford, 1965). In our studies a pH of 6·3 to 6·7 in the supernatant in infected cell cultures by the sixth day after inoculation has not affected the viability of cell-associated and extracellular mycoplasmas. This suggests that the organism in cell association is protected from the alkali-acid pH change in the supernatant fluid.

Morphological aspects

Examination of the stained preparations of the WI–38 cell cultures infected with mycoplasma showed that the mycoplasmas were located on the cell surface and probably inside the cells. The number of cell-associated mycoplasmas varied with the multiplicity of infection and with time of inoculation from few individual micro-organisms to heavy confluent masses in the cytoplasm and in the intercellular spaces. Disintegration of infected cell monolayers also varied with the multiplicity of infection and with time of incubation. On some occasions the whole of the cell monolayers would disintegrate by the seventh day but on other occasions it remained completely intact even after infection of a high multiplicity.

The infected cells showed morphological changes on the first examination, i.e. as early as 24 hr., with the appearance in a few cells of slight granulation in the cytoplasm; by 48 hr. more definite granulation appeared, mainly at the cell border and cellular processes. After the fourth day of infection large areas of the cell monolayer showed granulation of the cytoplasm and loss of cells was beginning to appear. The cell destruction never occurred before the maximum level of mycoplasma growth had been reached, i.e. day 4 to 5 after infection. To conclude, the morphological changes in our experiments were in all essentials similar to those observed by Fogh & Fogh (1964, 1967) with different mycoplasma-cell systems.

DISCUSSION

It has been known for a long time that mycoplasmas may be closely attached to the living cells so that they cannot be separated from them. Though discussed widely, the problem of the mode of mycoplasma-cell association has not yet been resolved. It may be that mycoplasmas may grow only on the surface of cells and in the intercellular spaces and their appearance in the cytoplasm may be the result of pinocytosis. Alternatively, they may be capable of intracellular residence in which they may assume a stage that allows survival only in association with the host cell. A clear understanding of the mode of mycoplasma-cell association will obviously have to await better knowledge of this group of microbes.

The main purpose of this study was to investigate the quantitative aspects of the growth of cell-associated and extracellular forms of *M. pneumoniae* in cultures of lung diploid fibroblasts as represented by the WI–38 cell strain. With this aim in mind we purposely selected a well-known laboratory strain (FH) of *M. pneumoniae* with a history of many passages in acellular media to attempt to answer the question whether or not these have reduced the original capacity of the micro-organism to invade living cells. The fact that this strain was never, to the best of our knowledge, passaged in cell cultures was a further inducement in these studies.

As reported in this paper, the WI–38 cell strain was shown to be a sensitive, susceptible host for the FH mycoplasma strain. The pattern of mycoplasma replication in cell cultures was quite specific in all experiments and the detectability and increases of cell-associated and extracellular mycoplasmas appeared to be closely related to the multiplicity of cell infection. At relatively high multiplicity levels

there was no substantial quantitative difference between the cell-associated and extracellular mycoplasmas in regard to the amount and the time of appearance. In contrast, with lower multiplicity it was found that the cell-associated mycoplasmas were preponderant and at the multiplicity of cell infection as low as 0·00007, i.e. 1 mycoplasma per 14,000 cells, only the cell-associated mycoplasmas were detectable and then only after a lag period of 5 days.

The puzzling problem of the occult period which occurs in cell infections of lower multiplicity deserves further study. It seems that during this period mycoplasmas cannot be recovered from infected cell cultures using the acellular medium, in which they have been propagated before their contact with living cells. This may be explained in various ways. It may be that the attachment to cell receptors (Taylor-Robinson & Manchee, 1967) and the process of cell pinocytosis weakened mycoplasmas in the homogenate and they temporarily lost the capacity to divide in acellular medium. Alternatively, they may have temporarily assumed a stage which allows survival only in association with living cells (Klieneberger-Nobel, 1962). That cell attachment involves an active biological process on behalf of either cell or mycoplasma has recently been convincingly demonstrated with different mycoplasma-cell systems (Fogh & Fogh, 1967; Taylor-Robinson & Manchee, 1967).

On the present evidence it seems possible that the occult period observed in our experiments (Table 1, Fig. 1) may have been due to a specific association between mycoplasmas and infected cells in culture. The possibility that the cells have provided only a convenient inert surface seems unlikely; if this would be the case, then mycoplasmas should have been recovered from cells infected at low multiplicity levels (Table 1) in the way they have been recovered from the inocula, i.e. without any occult period.

The origin of the increasing extracellular mycoplasmas in infected cell cultures is a difficult but also a most interesting question. It is quite certain that SM 199 alone or after supplementation with the WI–38 cell extract does not support the growth of *M. pneumoniae*. It cannot be ruled out, of course, that the infected cell cultures may produce some substances which enable mycoplasmas to grow in the SM 199 supernatant. On the other hand, it is clear from our experiments with lower multiplicity of infection that evidence for such assumption is lacking. It is obvious, therefore, that most extracellular organisms were produced in association with infected cells, being liberated into the supernatant from the partly destroyed cytoplasm.

It must be pointed out that this investigation was limited to one strain of mycoplasma and one type of cell culture, and it is quite possible that other strains may show different patterns of their growth in different cell cultures. Thus each strain may pose a separate problem. A further problem is the sensitivity of the method using acellular media for recovery from infected cells of mycoplasmas which have temporarily assumed a stage which allows survival only in association with living cells.

Although the mode of mycoplasma-cell interaction has not yet been settled, it is already clear that mycoplasmas are not easy to kill in cell environment without also killing the cell. With regard to eradication of mycoplasmas from contaminated cell cultures, the most recent reviews indicate that, regardless of the source of the

initial contamination, there is no effective decontamination method which is universally applicable (Hayflick & Chanock, 1965; MacPherson, 1966). Some drastic methods which were reported as successful for decontamination of cell cultures could not of course be used for eradication of mycoplasmas in the body.

That antibiotics can penetrate mammalian cells is clearly shown in a review by Collins (1965). Much less is known, however, of the probability of a circulating antibiotic crossing a number of barriers that separate it from an intracellular parasite (Kessel, 1965). It appears, then, that caution has to be exercised in the determination of the potency of antibiotics against mycoplasmas by methods employing acellular media alone, and it is desirable to utilize also appropriate cell culture models. A possible approach to designing such models is provided by this study.

SUMMARY

The FH strain of *Mycoplasma pneumoniae* was studied in experimentally infected cultures of lung diploid fibroblasts. The multiplicity of infection was found to have a profound influence on the distribution of the cell-associated and extracellular mycoplasmas. At a high multiplicity there was very little difference between the ratio of cell-associated and extracellular mycoplasmas. In contrast, with a low multiplicity the cell-associated mycoplasmas predominated and with further reductions of the inoculum only the cell-associated mycoplasmas could be detected. The significance of these findings is discussed. It is suggested that caution has to be exercised in the determination of the potency of antibiotics against mycoplasma by methods employing acellular media alone, and it is desirable to utilize also appropriate cell culture models. A possible approach to designing such models is provided by this study.

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