THE BEHAVIOUR OF T-MYCOPLASMAS IN TISSUE CULTURE

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PLATE IX

MYCOPLASMAS were first isolated from tissue-culture cells by Robinson, Wichelhausen and Roizman (1956). Since that time they have been isolated from a variety of tissue cultures, particularly continuous cell lines (Macpherson, 1966). T-mycoplasmas were first isolated from the human genital tract by Shepard (1954), and since then from various animal specimens also (Taylor-Robinson, Williams and Haig, 1968; Taylor-Robinson *et al.*, 1971). In man they are primarily associated with the genital tract (Shepard *et al.*, 1964; Taylor-Robinson *et al.*, 1969), but are present also in the mouth of about 5 per cent. of persons (Taylor-Robinson and Purcell, 1966; Purcell *et al.*, 1967; Ford, 1967; Shepard, 1970). There would, therefore, seem to be opportunities for contamination of tissue cultures by T-mycoplasmas to occur. Nevertheless, there are no reports of this having happened; this could be due to an inability of T-mycoplasmas to survive or to a failure to examine cultures adequately for such contamination. We have examined cultures, and also continuous cell lines experimentally infected with T-mycoplasmas and compared the growth of these organisms with that of other mycoplasmas.

MATERIALS AND METHODS

Tissue-culture cells. L132 cells: a continuous line of human embryo lung cells (Davis and Bolin, 1960) obtained from the American Type Culture Collection. HeLa cells: rhinovirus-sensitive cells obtained from Dr V. V. Hamparian, Ohio State University, USA (Conant and Hamparian, 1968). Vero cells: a continuous line of African green monkey kidney cells (Yasumura and Kawakita, 1963) obtained from the American Type Culture Collection. WI-38 cells: a diploid strain of human embryo lung fibroblasts (Hayflick and Moorhead, 1961).

Mycoplasmas. Two large-colony-forming mycoplasmas were used, namely *M. hominis* isolated in this laboratory from a throat swab and passaged only twice in artificial medium, and *M. orale* type 1 isolated in this laboratory from HeLa cells. Two T-mycoplasmas were used: the first, designated SP1467, was isolated from a patient suffering from post-gonococcal, non-specific urethritis and was cloned and passaged twice in mycoplasma liquid medium; the second, designated "Johnson", was isolated from the mouth of an apparently healthy person (Taylor-Robinson and Purcell), and was cloned and passaged four times in mycoplasma medium.

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Media. (i) *For tissue-culture cells.* The cells were grown in Eagle's basal medium (BM) containing 10 per cent. unheated ox serum and 1000 units of penicillin per ml (pH 7.0). They were maintained in the same medium but with 2 per cent. unheated ox serum. In addition, 0.01–0.05 per cent. urea and 0.05–0.1M N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) buffer (Williamson and Cox, 1968) were added to some batches of maintenance medium, which were then adjusted to pH 7.0 with 0.2M NaOH. (ii) *For mycoplasmas.* Large-colony-forming mycoplasmas were isolated and grown in a medium described previously (Manchee and Taylor-Robinson, 1968), which basically consisted of Difco PPLO broth supplemented with 2.5 per cent. yeast extract, 20 per cent. unheated horse serum and bacterial inhibitors; solid medium was the same with the addition of 1 per cent. Ionagar no. 2. T-mycoplasmas were grown in the same liquid medium to which was added 0.1 per cent. urea. The media contained 1000 units of penicillin per ml, but no thallium acetate.

Growth of tissue-culture cells. Growth medium was removed from confluent monolayers and for L132, Vero and WI-38 cells a solution of 0.125 per cent. (w/v) trypsin in pH 7.2 phosphate-buffered saline (PBS) containing 0.025 per cent. (w/v) ethylenediamine-tetraacetic acid (EDTA) was added. For HeLa cells, 0.05 per cent. (w/v) EDTA in PBS was used. After 5 min. at room temperature the cells were resuspended in fresh growth medium so that there were about $(0.05\text{--}1.0) \times 10^6$ cells per ml. Cells were grown as monolayers in $6 \times \frac{3}{8}$ in. (15×1.5 cm) tubes and 2 oz. flat-sided medicine bottles. The tubes and bottles were kept in a stationary position at 37°C for growth of cells and after inoculation of mycoplasmas.

Disruption of tissue-culture cells. Two methods were used to disrupt cells in order to free mycoplasmas from them: (i) resuspension in serum-free maintenance medium and agitation for 1 min. in an M.S.E. homogeniser set at maximum speed; (ii) resuspension in the same medium and freezing at -20°C and thawing at room temperature.

Detection of urea. The presence of urea in various samples was detected by placing half a tablet of urease (British Drug Houses) in 1 ml of the sample and adding 1 ml of Nessler's reagent. The development of a purple colour after a few minutes indicated the presence of urea. Attempts were also made to measure the urea content of samples on an autoanalyser.

Titration of mycoplasmas. Quantitative estimations of large-colony-forming mycoplasma organisms were made by counting colonies on agar medium. Similar estimations of T-mycoplasma organisms were done by making serial ten-fold dilutions in liquid medium containing 0.1 per cent. urea and phenol red indicator. Growth was detected by a change in colour of the medium from yellow (pH 7.0) to pink ($\text{pH} \geq 7.8$). The highest dilution which produced a colour change after continued incubation at 37°C was the end-point of the titration and this was considered to contain one colour-changing unit (CCU).

Treatment of T-mycoplasma-infected cells with streptomycin. Cells in monolayers and cells in suspension after EDTA-trypsin treatment were incubated at 37°C for 2 hr with maintenance medium that contained 100 µg per ml of streptomycin. The medium was then removed and fresh medium was added. Further details are presented in the *Results* section.

RESULTS

Technique of mycoplasma isolation

Before considering the growth of T-mycoplasmas in tissue culture, several continuous cell lines that were likely to be chronically infected by mycoplasmas were investigated to determine the best means of isolating these organisms.

Cultures of three different passages of HeLa cells (HeLa 1–3), HeLa cells after tylosin treatment (HeLa 4), L132, Vero and WI-38 cells were examined (table I). *M. orale* type 1 was isolated only from the different passages of HeLa cells untreated with tylosin. The culture medium contained the smallest number of viable organisms. Cells were removed from the glass surface by

EDTA-treatment and resuspended in the original volume of serum-free maintenance medium. A sample of this suspension usually contained more organisms, and disruption of the cells by freezing and thawing or by homogenisation led to the release of an even greater number of organisms. It was not possible to say whether this was due to liberation of organisms from on or within the cells, but clearly it was an important step in the isolation of this mycoplasma from these cells.

Experimental infection of L132 cells with large-colony-forming mycoplasmas

Because mycoplasmas were not isolated in numerous attempts from the continuous line of L132 cells we decided to use them for experimental infection (see

TABLE I
Isolation of mycoplasmas from various cell cultures

Sample examined	Number of colonies on agar after inoculation of sample from indicated cell type						
	HeLa (1)	HeLa (2)	HeLa (3)	HeLa* (4)	L132*	Vero*	WI-38
(1) Culture medium	2	100	100	0	0	0	0
(2) Suspension of EDTA- or trypsin-treated cells	400	100	100	0	0	0	0
Same suspension as (2), but after freezing and thawing	900	≥5000	1000	0	0	0	0
Same suspension as (2), but after homogenisation	...	...	1000	0	0	...	...

* Used in subsequent mycoplasma infection experiments.

Discussion). The growth of two large-colony-forming mycoplasmas, *M. orale* type 1 and *M. hominis* was first studied. *M. orale* type 1 grew slowly, an increase in the number of organisms in the medium of infected cultures not being detected before 7 days. *M. hominis* grew rapidly, there being at least a 100-fold increase in the number of organisms after 3 days, and there was evidence of its continued but diminishing presence for over a month (fig. 1). With this mycoplasma, organisms were isolated in greater numbers from the maintenance medium of infected cultures, which was unchanged throughout the experiment, than from the disrupted cells. Maintenance medium from tubes without cells did not support growth.

Experimental infection of cell cultures with T-mycoplasmas

Cultures of L132 cells were most frequently used, but occasionally Vero and HeLa cells from which mycoplasmas could not be isolated were also used.

Urea requirement. Cultures of L132, Vero and HeLa cells, some maintained in medium containing additional urea, were inoculated with T-mycoplasmas SP1467 and "Johnson". At various times thereafter the media were titrated for viable organisms. The results for each type of cell system were similar and those obtained for L132 cells are presented in fig. 2. The addition of 0.05 per cent. urea to the medium stimulated mycoplasma growth. In the presence of urea there was a 1000-fold increase in the number of viable organisms of the "Johnson" mycoplasma, but no growth occurred in the absence of urea. The number of viable organisms of T-mycoplasma SP1467 was, however, only ten-

FIG. 1.—Growth of *M. hominis* in L132 cell cultures. ○—○ = Maintenance medium from cell culture; ▲—▲ = frozen and thawed cells; ●---● = homogenised cells; ⊗—⊗ = maintenance medium from a tube without cells.

fold less in medium without urea. Clearly, T-mycoplasma "Johnson" was more dependent on urea for growth than was T-mycoplasma SP1467.

Growth in maintenance medium from tubes without cells. The results of a previous experiment with *M. hominis* (*vide supra*) showed that maintenance medium from tubes without cells did not support the growth of this mycoplasma. In contrast, however, T-mycoplasma SP1467 sometimes grew in cell-free maintenance medium, i.e., containing 2 per cent. ox serum. There was a 100-fold increase in the number of viable organisms after the addition of 0.05 per cent. urea to this medium (fig. 3), and T-mycoplasma "Johnson" grew only in the urea-containing medium.

Growth in association with cells. The question arose of whether the growth of mycoplasmas that was detected in the whole culture (fig. 2) was entirely due to multiplication of the T-mycoplasmas in the maintenance medium or whether

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FIG. 5*a*. FIG. 5*b*.

FIG. 5.—Cytopathic effect produced by T-mycoplasma SP1467 in L132 cell cultures: (*a*) uninoculated culture after 36 hr at 37°C in maintenance medium containing 0.05 per cent. urea; (*b*) SP1467-inoculated culture after 36 hr at 37°C in maintenance medium containing 0.05 per cent. urea. Single and groups of rounded refractile cells are to be seen.

FIG. 6*a*. FIG. 6*b*.

FIG. 6.—Cytopathic effect produced by T-mycoplasma SP1467 in HeLa cell cultures: (*a*) uninoculated culture after 36 hr at 37°C in maintenance medium containing 0.05 per cent. urea; (*b*) SP1467-inoculated culture after 36 hr at 37°C in maintenance medium containing 0.05 per cent. urea. Large round cell and multivacuolated cell arrowed.

FIG. 2.—Growth of T-mycoplasmas in L132 cell cultures. ●—● = Maintenance medium containing 0.05 per cent. urea from "Johnson"-infected cell cultures; ○—○ = maintenance medium from "Johnson"-infected cell cultures; △---△ = maintenance medium containing 0.05 per cent. urea from SP1467-infected cell cultures; ▲---▲ = maintenance medium from SP1467-infected cell cultures.

FIG. 3.—Growth of T-mycoplasma SP1467 in tubes with and without L132 cells. ■—■ = Maintenance medium containing 0.05 per cent. urea from tubes with L132 cells; ⊗—⊗ = maintenance medium from tubes without cells; △---△ = maintenance medium containing 0.05 per cent. urea from cell cultures; ▲---▲ = maintenance medium from cell cultures.

the cells contributed to their growth. Several experiments, in which maintenance medium alone was infected as well as cell cultures, indicated that multiplication of organisms was greater in the presence of cells than without cells (fig. 3), particularly in the absence of urea.

In a further experiment (fig. 4), L132 cells were maintained in maintenance medium without serum. This medium did not itself support the growth of *T-mycoplasma* SP1467, but this mycoplasma grew in cell cultures maintained in the serum-free medium. A similar result was obtained with *T-mycoplasma* "Johnson". Cells from the serum-free cultures produced a colour change in a qualitative test for urea, but the amount of urea was too small to be measured on an autoanalyser. These results supported the contention that the tissue

FIG. 4.—Growth of *T-mycoplasma* SP1467 in L132 cell cultures maintained without serum. ●—● = Maintenance medium containing 0.05 per cent. urea from cell cultures; ○—○ = maintenance medium from cell cultures; ▲—▲ = frozen and thawed cells from cultures without urea; ■—■ = frozen and thawed cells from cultures containing 0.05 per cent. urea; ⊗—⊗ = maintenance medium from tubes without cells.

cells, and probably the urea within them, contributed to the growth of the *T-mycoplasmas*.

Whether the organisms were closely cell-associated or grew mainly in the overlying maintenance medium of infected cultures was also investigated. The results (fig. 4) showed that the greatest number of *T-mycoplasma* organisms was present in the culture medium, particularly in medium with additional urea. Fewer organisms were found in close association with the cells, in this case the greatest number being detected and persisting for a longer time in the absence of urea in the medium. Similar results were obtained when the culture medium contained serum. In addition, cells previously disrupted by freezing and thawing or those treated at 56°C for 30 min. did not support growth of either *T-mycoplasma*.

Intracellular or extracellular existence of T-mycoplasmas

Several experiments were done to determine whether the T-mycoplasmas that were in close association with the tissue-culture cells were within them or on their surfaces.

Effect of streptomycin on T-mycoplasmas. 10^6 CCU of T-mycoplasmas SP1467 and "Johnson" in mycoplasma liquid medium were incubated at 37°C for 2 hr with $100\ \mu\text{g}$ streptomycin per ml. This concentration of antibiotic was lethal: no viable organisms were recovered after this time.

TABLE II

Isolation of T-mycoplasmas from streptomycin-treated L132 cells

Type of culture	T-mycoplasma	Expt. no.	Log ₁₀ CCU of viable mycoplasmas per ml			
			before streptomycin treatment in		after streptomycin treatment in	
			culture medium	frozen cells	medium before freezing	frozen cells
Monolayer	SP1467	1	6	5	0	5
		2	6	5	0	4
	"Johnson"	3	4	4	0	2
Suspension	SP1467	1	6	5	0	5
		2	5	4	0	4
	"Johnson"	3	4	4	0	3

Effect of streptomycin on infected tissue-culture cells. Monolayer cultures of L132 cells in maintenance medium were infected with T-mycoplasmas SP1467 and "Johnson". After incubation for 24 hr at 37°C , the medium was removed and titrated for viable organisms. All the cultures were washed three times with medium; some of them were frozen and thawed and the medium was titrated, and to others fresh medium containing $100\ \mu\text{g}$ per ml of streptomycin was added. After incubation for 2 hr, the streptomycin-treated cultures were washed again three times and medium without streptomycin was added. This medium was titrated for viable organisms; the cells were then frozen and thawed and the medium was again titrated. The results are shown in table II. After streptomycin treatment the organisms were recovered only from disrupted cells, in which they were present in numbers similar to those obtained from cells before treatment.

In a further experiment, L132 cells were infected while suspended in maintenance medium. The cells were treated in a manner similar to that described above except that they were centrifuged at $600g$ for 10 min., resuspended in medium which contained streptomycin and recentrifuged before freezing and

thawing. The results (table II) are similar to those of the previous experiments with cells in monolayers and indicate that the organisms can become intracellular.

Cytopathic effect produced by T-mycoplasmas

Cytopathic changes were observed in some of the monolayer cultures maintained in medium containing urea after inoculation of T-mycoplasmas (table III). The changes were not very striking and occasionally disappeared on continued incubation; it is possible that this could account for our failure to observe a change in L132 cells inoculated with T-mycoplasma "Johnson". Single cells and groups of a small number of rounded refractile cells developed in L132 cells between 24 and 36 hr after inoculation of 10^3 CCU of T-mycoplasma SP1467 (fig. 5a and b). Changes in HeLa cell cultures inoculated with either T-mycoplasma SP1467 or "Johnson" consisted of the formation of a

TABLE III

Occurrence of cytopathic effect (CPE) produced by T-mycoplasmas in various cell cultures

T-mycoplasma	Presence (+) or absence (—) of CPE in indicated cell-type			
	L132	HeLa	Vero	WI-38
SP1467	+	+	+	—
"Johnson"	—	+	+	—

few multivacuolated giant cells and single, diffusely scattered, rounded refractile cells (fig. 6a and b). Such changes were observed also 24–36 hr after inoculation. Changes in cultures of Vero cells again consisted of round refractile cells which were distributed singly or in small groups; these changes did not occur before 72 hr—i.e., later than those observed in the other types of cells. Less striking changes were seen in cultures of L132 cells maintained in medium without urea and inoculated with T-mycoplasma SP1467. Furthermore, no changes were seen in similarly maintained cultures of HeLa and Vero cells.

L132 cells were suspended in growth medium, infected with T-mycoplasma "Johnson" and then allowed to form monolayers. A greater number of vacuolated cells was seen after 48 hr than in uninfected control cultures.

Attempts to maintain a T-mycoplasma infection of tissue-culture cells

L132 cells maintained in medium without urea and infected with T-mycoplasma SP1467 were subcultured 24, 36, 48 and 72 hr after inoculation. At 36 hr, immediately before subculture, the titre of organisms in the maintenance medium was 10^5 CCU per ml. After passage, mycoplasmas were recovered only from those cells that were subcultured at 36 hr. T-mycoplasmas were maintained in this culture during two further passages, each of which was done at an interval of 36 hr; in each case, the titre of viable organisms immediately before subculture was 10^3 CCU per ml of growth medium and 10^5 CCU per ml

of growth medium containing frozen and thawed cells. Unfortunately, continued subculture had to be abandoned because passage at such frequent intervals led to a diminution in the number of viable cells.

Growth of T-mycoplasmas in "conditioned" medium

Growth medium removed from cultures of L132 cells after 2 days of incubation did not support growth of T-mycoplasma SP1467, whereas the medium removed from cultures of Vero and HeLa cells did support growth (fig. 7). This suggested that an inhibitor might be present in the cultures of L132 cells.

FIG. 7.—Growth of T-mycoplasma SP1467 in "conditioned" media removed from cultures of various cell-type after incubation at 37°C for indicated no. of days. ○---○ = Medium from L132 cells after 2 days; ○—○ = medium from Vero cells after 2 days; ▲—▲ = medium from HeLa cells after 2 days; ●—● = medium from Vero cells after 11 days; ▼—▼ medium from HeLa cells after 13 days; ■—■ = medium from tubes without cells after 2 days.

Medium removed from cultures of L132 cells after 2 days of incubation and added to fresh medium containing 10⁴ CCU of T-mycoplasma SP1467 organisms inhibited their multiplication. This further confirmed the presence of an inhibitor. An apparently less active inhibitor was present also in cultures of Vero and HeLa cells, because media removed from these after 11–13 days of incubation inhibited growth of the T-mycoplasma (fig. 7).

DISCUSSION

Shepard (1954) first isolated T-mycoplasmas from the genital tract of man. He reported then, and subsequently (Shepard, 1960, 1967, 1969, 1970), that these organisms were closely associated with epithelial cells present in inflammatory exudates from the urethra. To explore further the relationship of

T-mycoplasmas with cells, Shepard (1958) inoculated HeLa cell cultures. At this particular time, however, there were considerable difficulties in growing T-mycoplasmas, and the material used as inoculum had been egg-passaged. It appears that an avian mycoplasma, *M. gallisepticum*, contaminated the inoculum and, as Shepard (1969) has subsequently pointed out, the results are difficult to interpret. Our results show that it is possible to infect various tissue-culture cells with T-mycoplasmas and that they produce a minimal cytopathic change. We do not believe, however, that this should necessarily be interpreted as indicating that they behave in this way in the natural host and are a cause of disease. The evidence that they cause disease in man (Shepard *et al.*, 1964; Csonka, Williams and Corse, 1966; Shepard, 1970) has been strongly questioned (Ingham *et al.*, 1966; Black and Rasmussen, 1968; Fowler and Leeming, 1969; Hare, Dunlop and Taylor-Robinson, 1969). Furthermore, other mycoplasmas, for which there is no evidence of pathogenicity, are capable of producing even more profound changes in tissue cultures than T-mycoplasmas do.

Many of our experiments were done with cultures of L132 cells. These cells are particularly sensitive to a number of respiratory viruses (Bradburne, 1969). We used them because of their easy availability and because we failed repeatedly to isolate contaminating mycoplasmas from them. If such contamination did exist, the organisms were present in small numbers only and, we feel, are not likely to have interfered with infection by the T-mycoplasmas. In T-mycoplasma-infected cell cultures, the cells and the medium have to be considered. It is clear that the cells are concerned in the growth of T-mycoplasmas because tissue-culture cell maintenance medium devoid of serum does not support the growth of the organisms, but it will do so in the presence of cells. This, however, is an artificial situation, because serum is required for growth and often for maintenance of cells. In the presence of serum T-mycoplasmas will sometimes grow in cell maintenance medium that has not been in contact with cells, unlike the large-colony-forming mycoplasmas that we tested. However, the T-mycoplasma growth cycle, whether or not growth occurs in the culture medium or in close association with the cells, is short, being not longer than 48–72 hr. Organisms are not recoverable after this time. This period is shorter than the optimal period required for subcultivation of the cells, so that even though a special effort may be made to establish a chronic infection by T-mycoplasmas, it is self-limiting. It seems that a mycoplasma inhibitor develops in the cell cultures, particularly in those of L132 cells, and this may be a contributory factor in the failure to set up a chronic infection. It is of interest that the short duration of infection is in contrast to the apparently more persistent infection that occurs in the natural host and in organ cultures of human fallopian tube and bovine embryo trachea and urethra (Taylor-Robinson and Reed, unpublished).

A characteristic feature of T-mycoplasmas is their unique ability to metabolise urea (Purcell *et al.*, 1966; Shepard, 1966). Indeed, Shepard and Lunceford (1967) and Ford and MacDonald (1967) have shown that the presence of urea in cell-free mycoplasma medium is an important and possibly essential

requirement for the growth of these organisms. It seems that there are quantitative differences in the requirements for urea, since in our experiments T-mycoplasma "Johnson" grew in maintenance medium from tubes without cells only after the addition of urea, whereas T-mycoplasma SP1467 grew without additional urea, probably because urea was present in the serum. The presence of cells in serum-free culture medium led to some T-mycoplasma growth even though urea was not added. However, urea was not completely absent from these cultures, very small amounts being present probably within the cells. The addition of urea to the serum-free culture medium enhanced the growth of T-mycoplasmas. Clearly urea is important and organisms in close association with the cells may metabolise small amounts. Of course, other growth factors may be derived from the cells too. There was no evidence that a very low multiplicity of infection led to the establishment of cell-associated organisms only, as occurred in *M. pneumoniae* infection of human lung diploid fibroblasts (Larin, Saxby and Buggey, 1969).

There was an increase in the number of large-colony-forming as well as T-mycoplasma organisms, as judged by CFU or CCU, as a result of freezing and thawing or homogenising cells. This suggested to us that the organisms might be associated not only with the surface of the cells but also have an intracellular existence at some stage. This, of course, is a controversial problem that has been considered previously on numerous occasions. Freundt (1959) mentioned that no investigator had demonstrated the existence of an obligatory or even fairly frequently occurring intracellular mycoplasma growth-phase. Others (Shepard, 1956; Hayflick and Stinebring, 1955, 1960) suggested that the presence of cytoplasmic inclusions denoted an intracellular growth phase. This may be so, but it is difficult to decide whether the inclusions are mycoplasma elementary bodies or are caused by a metabolic product, or indeed whether they are on or within the cells. In our experiments on this point, we used streptomycin, which remains mainly extracellular (Brock, 1966). Furthermore, it had been shown previously (Taylor-Robinson, 1967) that low concentrations of streptomycin inhibited the growth of T-mycoplasmas, and we have shown here that they are rapidly lethal for T-mycoplasmas present in cell-culture maintenance medium. Therefore, our ability to recover these organisms from cells after treatment with this antibiotic in numbers similar to those present before treatment strongly suggests that the organisms become intracellular. Perhaps they are taken into the cells by a process of pinocytosis and are within vacuoles; *M. pulmonis* has been demonstrated within cytoplasmic vacuoles by electron microscopy (Hummeler and Armstrong, 1967). There is, however, no evidence that the T-mycoplasmas multiply within the cells. Our attempts to do similar experiments with antiserum rather than antibiotic failed because the antiserum was insufficiently potent to eliminate extracellular organisms completely.

SUMMARY

Mycoplasma orale type 1 and *M. hominis* increased in number in the maintenance medium of cultures of L132 cells. The former mycoplasma grew

slowly, but the latter grew rapidly and persisted in the cultures for more than a month. Neither mycoplasma grew in maintenance medium that had not been in contact with cells. In contrast, two T-mycoplasmas of human origin, one from the genital tract and the other from the oropharynx, grew rapidly in cultures of L132 cells and sometimes in maintenance medium from tubes without cells also, but persisted for 3–4 days only.

Even though there was growth of T-mycoplasmas in the maintenance medium of infected cell cultures, the cells contributed to the mycoplasma growth. Thus, serum-free maintenance medium alone did not support growth, but did so when used in cell cultures. Very small amounts of urea—probably derived from the cells—may have been responsible for the growth. The addition of 0.01–0.05 per cent. urea increased the number of viable T-mycoplasma organisms. In the presence or absence of additional urea, most organisms were found free in the maintenance medium overlying cell monolayers, but some were present in close association with the cells. Treatment of T-mycoplasma-infected cultures with streptomycin, which was lethal for extracellular T-mycoplasma organisms, followed by disruption of the cells, led to the release of viable organisms, indicating that some organisms were intracellular.

Minimal cytopathic changes were seen in cultures of L132, HeLa and Vero cells, particularly when 0.05 per cent. urea was added. Chronic infection could not be established in the cultures because the mycoplasmas usually died before the optimum time for cell subculture. The appearance of a T-mycoplasma inhibitor in the cultures possibly may have been partly responsible for this. The failure of T-mycoplasmas to persist in these cell cultures contrasts with their behaviour in the human host and in organ cultures.

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