Growth *in Vitro* of Mycoplasma-Infected Human Amnion Cells, FL Amnion Cells, and Mycoplasma-Modified FL Cells[1] (35371)

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It has been previously reported that the HT strain of mycoplasma fermentans (1) was highly cytopathic to cultures of FL human amnion cells (2). Morphological changes were observed as early as 1 or 2 days after infection, depending on the infecting dose. Since cultures of FL cells were more severely destroyed and showed more pronounced morphological changes after infection than many other cell lines examined (2), a number of the effects of this non-arginine dependent mycoplasma-human cell association has been investigated (3–9). The present report concerns observations of *in vitro* growth and morphology of mycoplasma-infected amnion cells, including mycoplasma-modified FL cells which have been reinfected with the mycoplasma.

Materials and Methods. Cells. Primary amnion cells, FL amnion cells (10), the mycoplasma-modified FL cell lines F 138 (1), still carrying mycoplasma, and F 138cl, F 159cl, and HTP8cl (1), representing mycoplasma-modified FL lines after mycoplasma elimination, were all cultured in LY medium (10) with 20% human serum, penicillin (100 units/ml), streptomycin (100 μg/ml).

Mycoplasma. Mycoplasma, strain HT, originally isolated as a tissue culture contaminant and identified as mycoplasma fermentans (PG-18), received many passages as an experimental contaminant of the cell lines derived from infected cultures of FL cells. For the present experiments, the mycoplasma was subcultured several times in BYE broth (11) to prevent the introduction of foreign cell types into the experimental cultures. The method for determining the titers of mycoplasma colony-forming units (CFU) has been described (4).

The presence, or absence, of mycoplasma in experimental cultures was demonstrated at frequent intervals by direct microscopic examination with phase optics of cell cultures exposed to hypotonic treatment, fixation, and orcein staining (12, 13). Quantitative determinations of cell-associated mycoplasma (4) were also based on this method.

Growth curve studies. Cells were removed from stock cultures by trypsinization. Tubes were inoculated with a constant number of cells per 2 ml of medium. The number of cells per culture was determined on trypsinized cell suspensions after various periods, using a hemacytometer for cell counts.

Cell morphology. The cell morphology was examined, in part, on living cultures by the interference-contrast method (Nomarski) with a Zeiss photomicroscope. They were grown on coverslips, and were mounted in a drop of LY medium with human serum by inverting the coverslip on a glass slide and by sealing the rim with a mixture of melted dental wax (75%) and Vaseline (25%). This rimming was necessary to prevent the formation of tiny bubbles which, in the absence of sealing, developed at the surface of many types of cells. Other cultures were examined after 15-min fixation in Bouin's solution and staining with hematoxylin and eosin.

Results. The difference between the growth *in vitro* of uninfected FL cells and cells of this line experimentally infected with the HT strain of *Mycoplasma fermentans*, can be illustrated by the growth curves in Fig. 1. As has been observed for other mycoplasma-infected FL lines, the time of population doubling was increased (FL: 17.5 hr; F 138: 30 hr). At the time of this experiment

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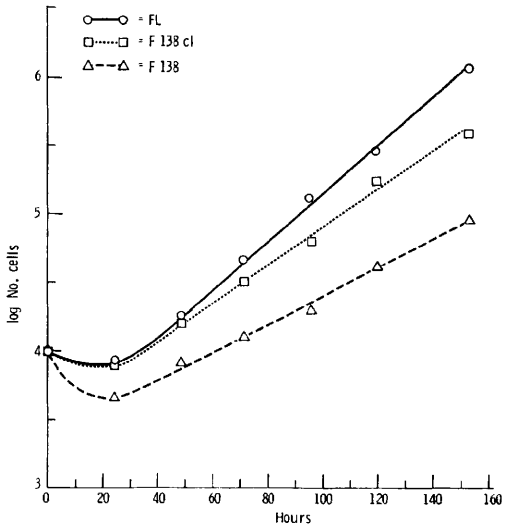


FIG. 1. Growth curves illustrating differences between the parent line of human amnion cells (FL); a mycoplasma-infected and -modified FL line (F 138) several years after the initial mycoplasma exposure; and the F 138 line after mycoplasma elimination (F 138cl).

cells of the mycoplasma-infected line (F 138) had been cultured serially for 1017 days. After elimination of mycoplasma (F 138cl) the doubling time decreased. Although 206 days had passed since mycoplasma elimination, this time (21 hr) was still longer than for the uninfected FL cells.

The initial response of FL cells to mycoplasma infection has been studied in many experiments (2), one of which is shown in Fig. 2. The growth rate was decreased, and from day 6 after infection there was also a loss of cells. During the following days, the combined increase in cell number and loss of cells resulted in a population at day 12 which was no more than that at day 2. In the third week the number of cells increased steadily. However, the rate of increase was considerably below that at which uninfected cells divided. When cells of the F 138cl line were infected with mycoplasma under similar conditions, the rate of increase in number was similar to the rate of infected FL cells for the first 6 days. From this time the reduction in cell number was much less (approx 10 times) pronounced than for FL cells. This difference in response to infection between FL and F 138cl cells has been confirmed in several following experiments. Two weeks after infection an increase in the number of cells at a rate similar to that for mycoplasma infected FL cells was observed.

The increased resistance of the F 138cl line, as compared to cells of the FL line, was also apparent from the different degrees of cytopathic effect and cell destruction seen in the two types of cultures after mycoplasma infection (Figs. 3–8). Cultures of both types of cells were seeded at similar density, and were compared 7 days after infection with similar inocula by interference-contrast microscopy of living cultures. The infected cultures of FL cells (Figs. 4 and 5) showed severe destruction of the cell sheet, debris, and a very light dense population of mostly spindle-shaped cells remained on the glass. In contrast, cell destruction in cultures of mycoplasma-reinfected F 138cl cells was only minor (Figs. 7 and 8); most cells maintained epithelial morphology, and spindle-shaped cells were rare. In a similar type of experiment, the response to mycoplasma infection was compared for FL cells and the two mycoplasma-modified lines F 159cl and HTP8cl. Again, the cell lines previously exposed to mycoplasma and subsequently carried in serial cultivation for several years in the absence

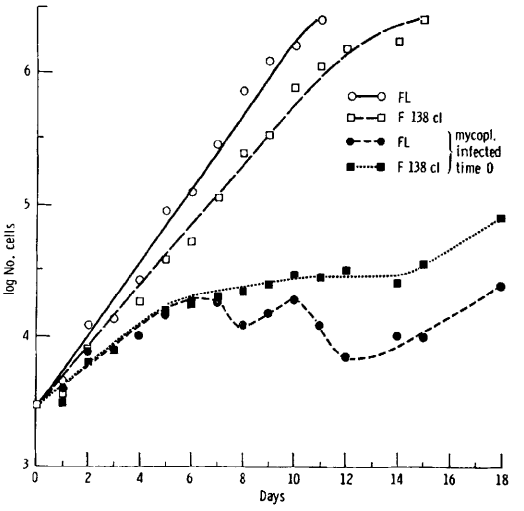
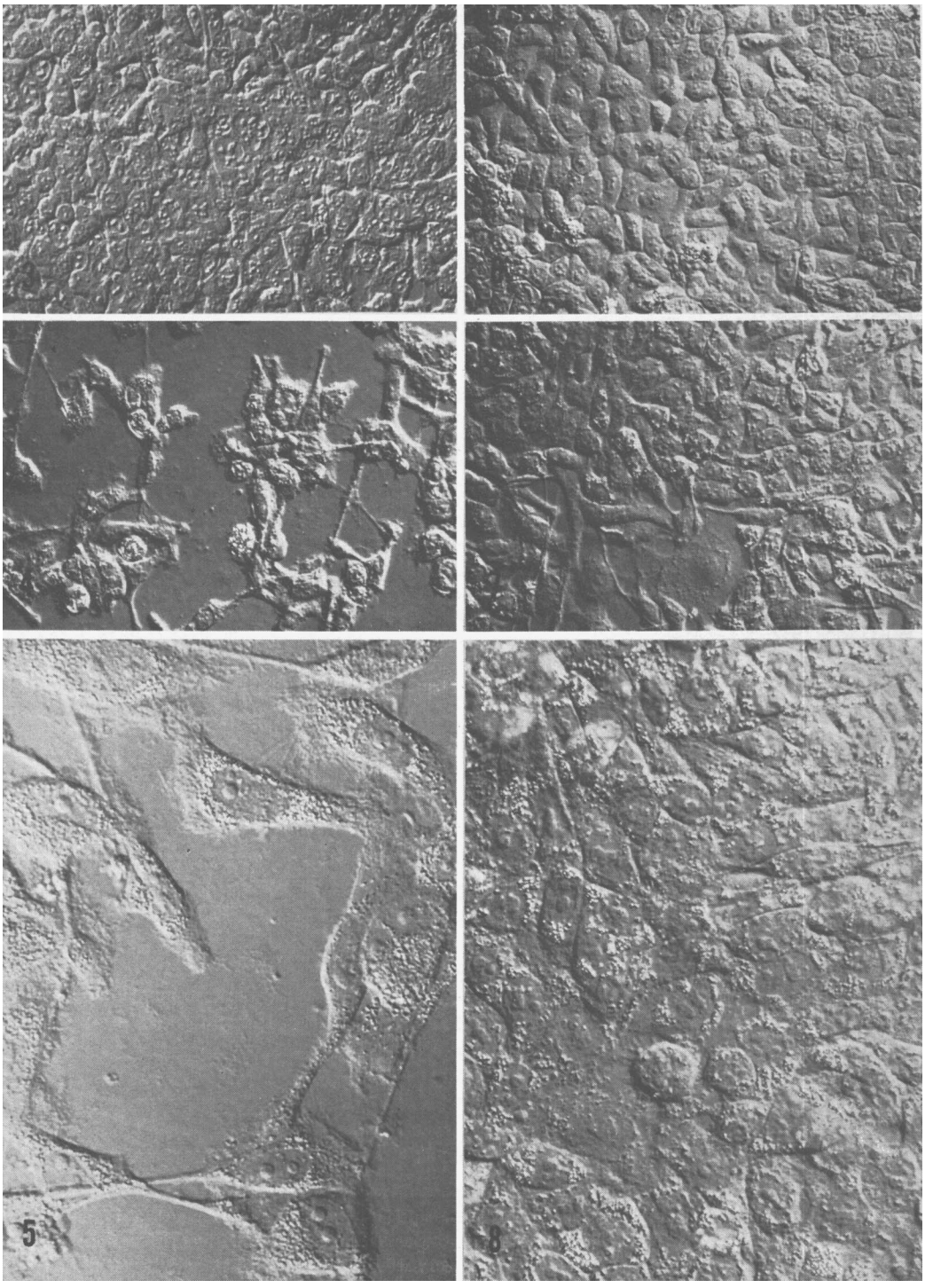
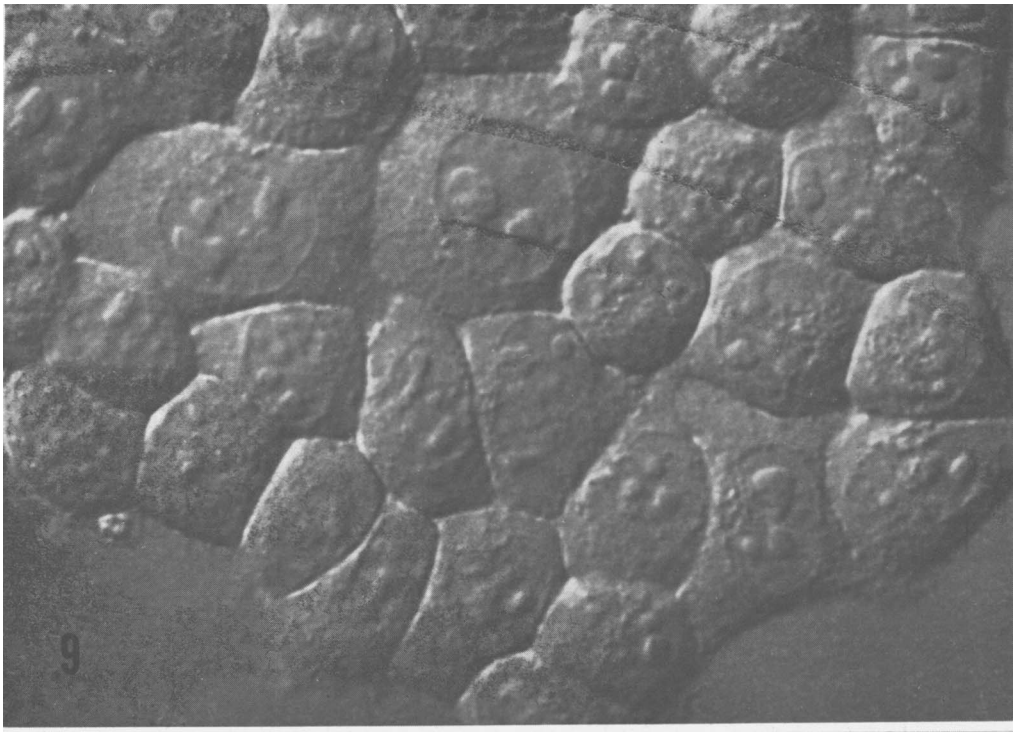
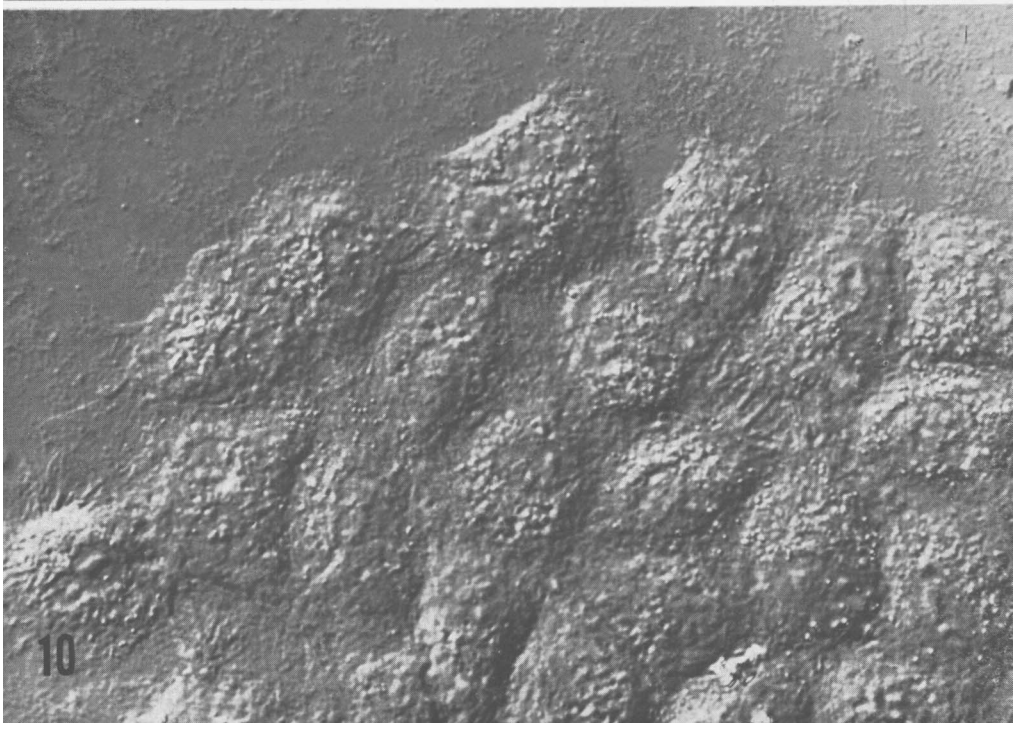


FIG. 2. Growth curves illustrating the different response of the FL and F 138cl line to infection with mycoplasma at time 0.

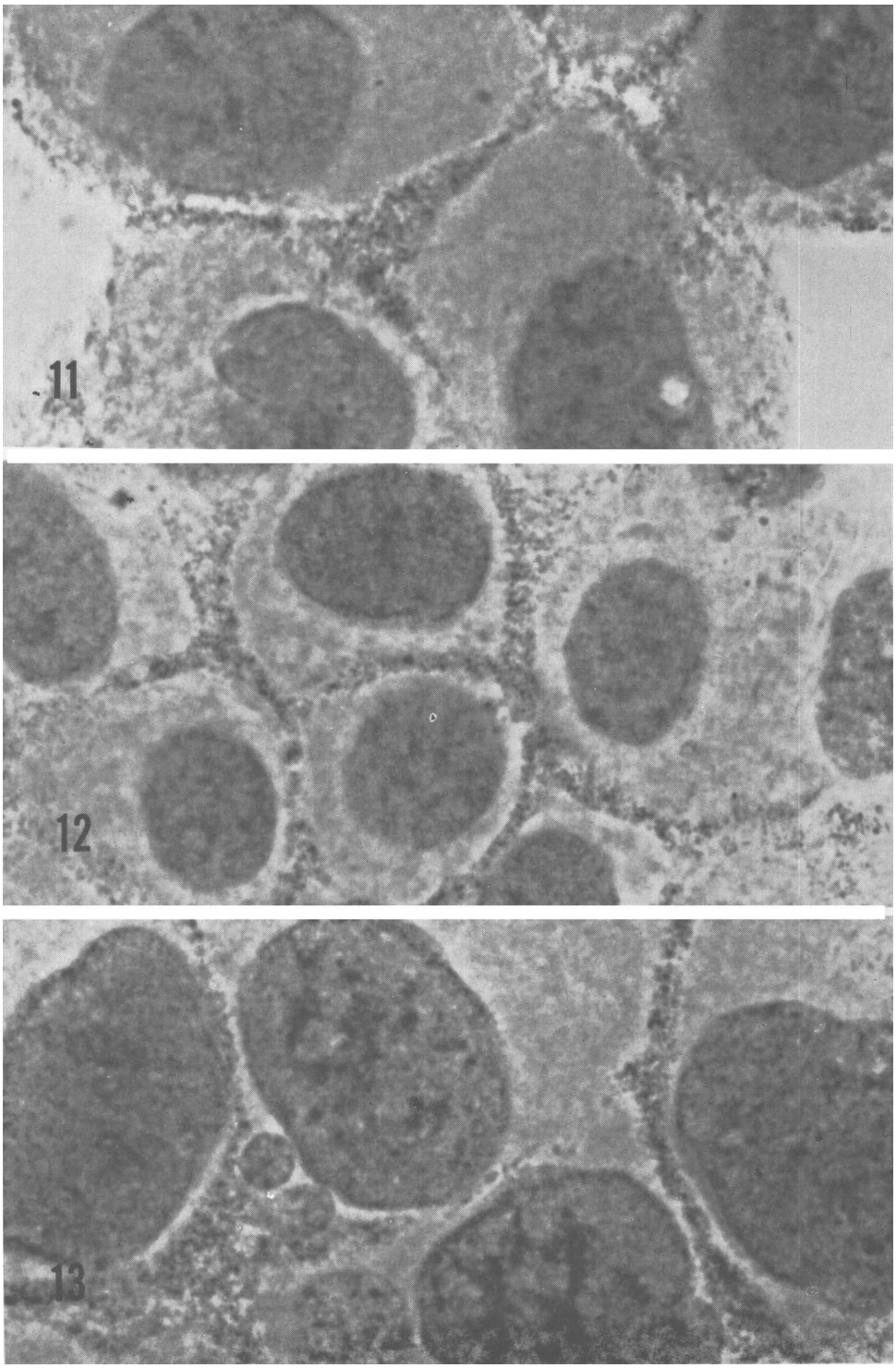


FIGS. 3–8. Differences in degree of cytopathic changes and cell destruction after mycoplasma infection of FL and F138cl cells: interference-contrast micrographs; Figs. 3, 4, 6, 7: 320×; Figs. 5 and 8: 800×. FIG. 3. Uninfected FL cells. FIGS. 4–5. FL cells 1 week after mycoplasma infection. FIG. 6. Uninfected F 138cl cells. FIGS. 7–8. F 138cl cells 1 week after mycoplasma infection.





FIGS. 9–10. The interference-contrast micrographs of living cells illustrate the distinct differences in appearance between uninfected FL cells (Fig. 9) and cells of the mycoplasma infected FL line (F 138) (Fig. 10). By this technique it is possible to distinguish quite clearly the mycoplasma, attached to the cells and glass; 1,280×.



FIGS. 11–13. Similar amounts of cell-associated mycoplasma are present after infection of the three cell types: FL (Fig. 11), F 138cl (Fig. 12), and F 159cl (Fig. 13); hypotonic treatment, Carnoy's fixation, orcein; phase optics; 2000×.

of mycoplasma, showed much less cell destruction.

The morphological differences between uninfected FL cells and cells of the F 138 line, mycoplasma-infected for several years, are also well demonstrated on living cultures by interference-contrast microscopy (Figs. 9 and 10). Cell-associated mycoplasma can be seen at high concentration on all F 138 cells. The cells, particularly the nuclei, appear to be smaller in size.

The relative resistance to reinfection of mycoplasma-modified FL cell lines from which mycoplasma were eliminated, conceivably could be the result of a reduced amount of cell-associated mycoplasma. However, this explanation does not appear to apply. The same degree of cell-associated mycoplasma was seen in cultures of FL cells (Fig. 11), F 138cl cells (Fig. 12) and F 159cl cells (Fig. 13) after mycoplasma infection under similar conditions, when the cultures were exposed to hypotonic treatment, fixation, and staining with orcein.

Cultures of primary human amnion cells were highly resistant to mycoplasma infection. The effects on cell density were examined by comparing the number of cells per infected and uninfected T-15 flask culture, 2, 4, and 6 weeks after infection at a multiplicity of 0.06 CFU/cell. The cultures were fixed and stained with hematoxylin and eosin, and cell counts were made in random areas using the photoframe built into the Zeiss photomicroscope. Mycoplasma infection effected no cell loss during this period (Table I). Morphological changes were inconspicuous at 2 and 4 weeks after infection. At 6 weeks the cells in the infected cultures were slightly more elongated than in the uninfected cultures. The difference between the response of primary amnion cells and FL cells was also clearly demonstrated by observing uninfected and mycoplasma-infected mixed cultures of FL and primary amnion cells after staining with hematoxylin and eosin. The size of FL cell colonies 1 week after infection was considerably reduced compared to similar uninfected cultures. Under these identical cultural conditions, there was evidence of selective and pronounced destructive effects of FL cells, whereas the morphology of the primary cells appeared unchanged (Figs. 14 and 15).

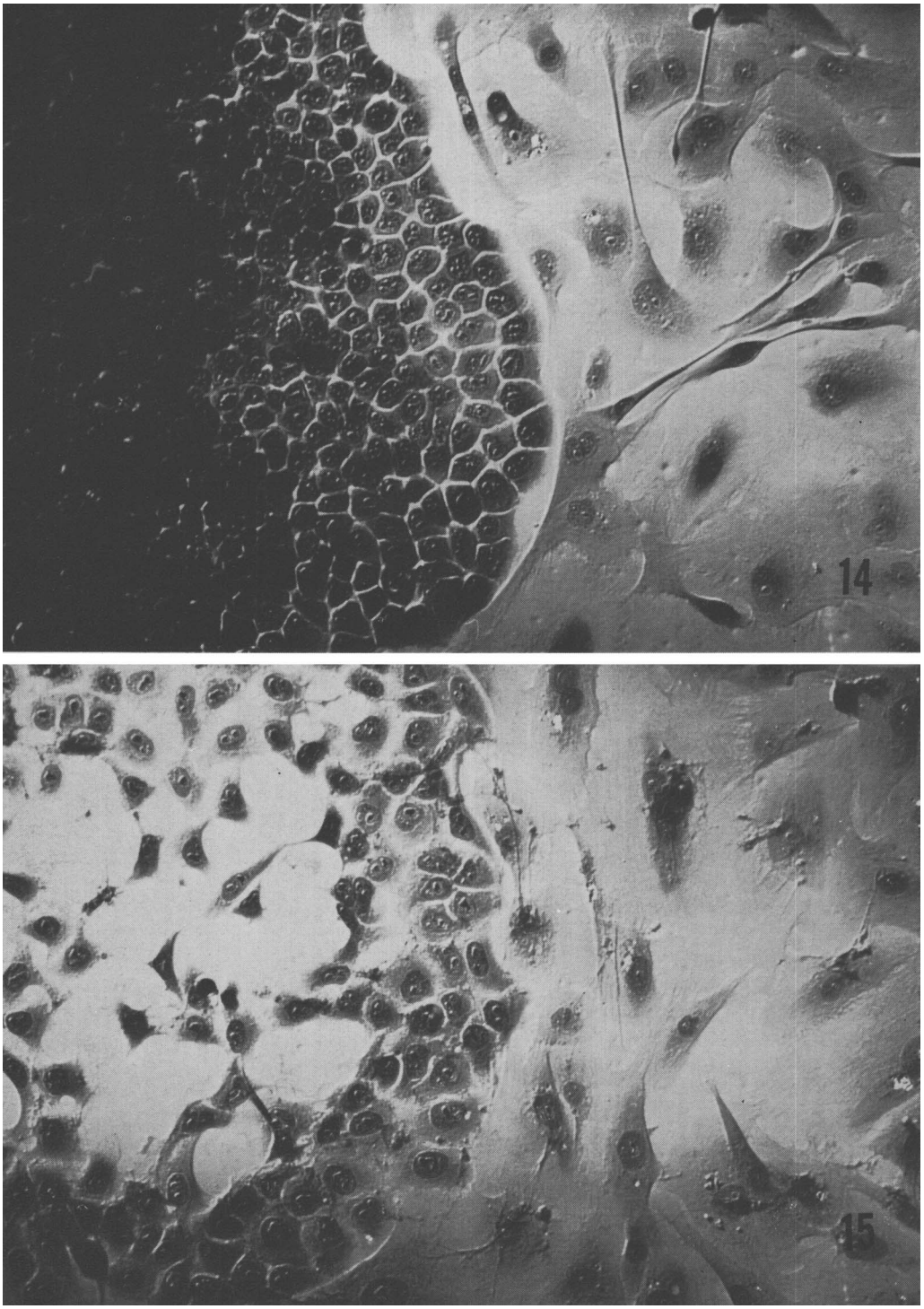
When FL cells were compared to primary amnion cells in mycoplasma-infected, mixed cultures, orcein-stained, cell-associated mycoplasma appeared to be present in considerably higher amounts on FL cells (Fig. 16). This seems to show that the mycoplasma selected the transformed cells to primary amnion cells for their propagation. A similar difference was observed in infected, mixed cultures of primary amnion and F 138cl cells. An increase was also observed in the mycoplasma content in the culture fluid from mixed cultures of primary amnion and FL cells compared to parallel cultures of primary amnion cells (Table I). It appears, therefore, that the mycoplasma propagates more vigorously in amnion cell cultures when FL cells are also present. Growth curves for this mycoplasma strain in cultures of FL cells have been published previously (7). Previous studies have also shown that mycoplasma titers increase with the number of FL cells per culture (4).

Discussion. The present data have demonstrated a pronounced resistance of primary amnion cells to mycoplasma infection, and

TABLE I. A. Number of Cells ($\times 10^6$)/Culture in Uninfected and Mycoplasma-Infected Primary Amnion (PA) Cell Cultures. B. Colony-Forming Units ($\times 10^6$)/ml of Mycoplasma in Supernatants of PA Cultures and in Comparable Mixed Cultures of PA and FL Cells.

0, 2, 4, 6 weeks after mycoplasma infection.

	Cell types	0	(weeks): 2	4	6
A	Inf. PA	8.1	7.2	7.1	6.2
	Uninf. PA	8.1	7.8	6.8	6.1
B	Inf. PA	(0.5)	20	3.3	8.2
	Inf. PA + FL	(0.5)	21	20	18



FIGS. 14–15. Interference-contrast micrograph of hematoxylin–eosin stained cultures of FL (left) and primary amnion cells. FIG. 14. uninfected; FIG. 15. mycoplasma-infected for 1 week. In the infected culture the morphology of the primary cells is intact; FL cells show cell destruction; 320×.

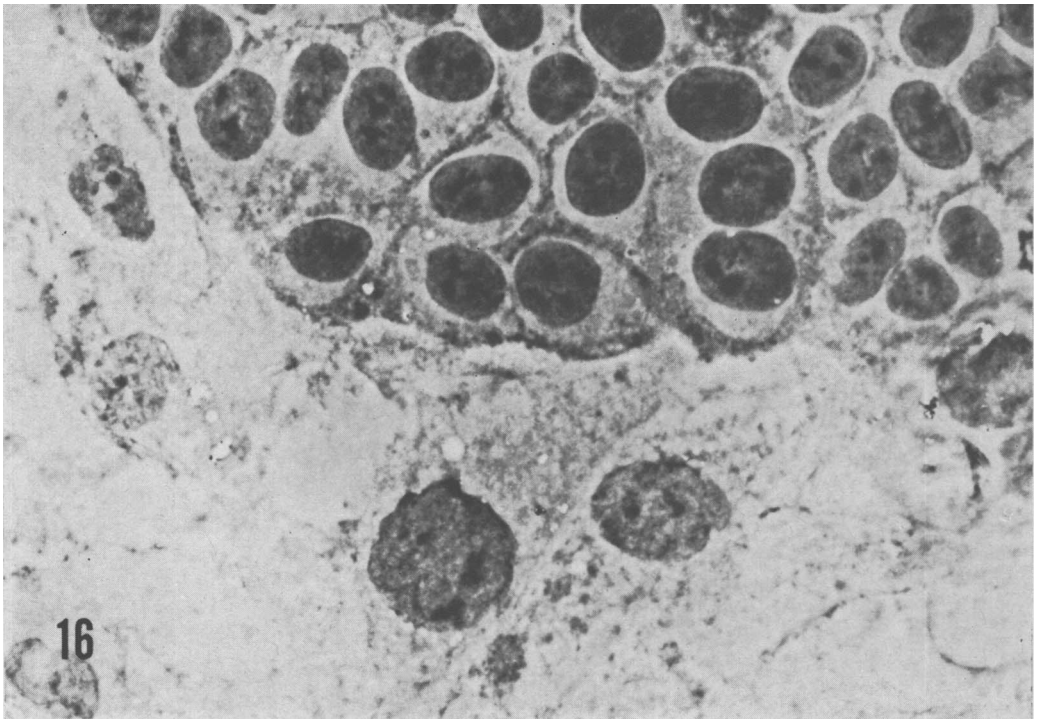


FIG. 16. Mycoplasma-infected culture of FL (top) and primary amnion cells. Mycoplasma are visible at high concentration in the intercellular spaces between FL cells. Primary amnion cells appear mostly free of mycoplasma. Hypotonic treatment, fixation, and orcein staining; phase optics; 800×.

have added to previously published data, several other differences between mycoplasma-modified FL cells and the parent FL cell line. An increased resistance to the cell destructive effects of mycoplasma, which can be observed early after infection of FL cells (2), is maintained after elimination of mycoplasma from the infected cell lines. This resistance can still be demonstrated several years after the mycoplasma elimination. The reduced *in vitro* rates of cell division of both the mycoplasma-infected lines and the lines from which mycoplasma were eliminated were also apparent several years after the infection. These rates parallel the rates of growth *in vivo* of the different lines (1). As compared to uninfected FL cells, the number of cells necessary for tumor production in the cheek pouch of cortisonized weanling hamsters was increased 5 to 10 times for the lines F 138, F 138cl, F 159cl, and HTP8cl; the tumor size was reduced and regression occurred earlier. Since these latter data were obtained 2 to 3.5 years after the original infection of FL cells, and up to 20 months after the mycoplasma elimination, and since mycoplasma-induced chromosome changes were also maintained several years after mycoplasma elimination (5, 1), both *in vitro* and *in vivo* expressions of these mycoplasma-induced changes must be considered to be irreversible. Various effects of mycoplasma on SV40 transformed human amnion cells also appeared to be stable (14, 15). Inheritable changes were observed when a line of hamster fibroblasts were infected with various strains of mycoplasma (16).

Since mycoplasma appear to be absent from the lines F 138cl, F 159cl, and HTP8cl, according to all tests employed, the increased resistance to infection of these lines and their changes in morphology and growth, must be interpreted as the results of a selection, an adaptation, or genetic changes, directly or indirectly induced by the original mycoplasma infection. Other observations

have supported the hypothesis that this infection may affect the cell genome (15); where and how this occurs is presently unknown. It is conceivable that one or more of the chromosome changes observed in this system (3) may be correlated with the phenotypic changes. The chromosome changes included a reduction in chromosome number, and the occurrence of several new chromosome varieties. There was also an increase in the frequencies of many abnormalities. After mycoplasma elimination, their frequencies decreased to the level observed in uninfected FL cells (5), but chromosome numbers remained decreased [although the amount of DNA was similar to that of FL cells (8)], and the new varieties were stable. Since primary amnion cells are highly resistant to mycoplasma infection, it is possible that the different resistance of the cell types presently studied reflect their difference in chromosome number (FL cells with the highest numbers were most sensitive; primary amnion cells with normal chromosome complement were most resistant).

Another difference, demonstrated by the present data, concerns the amounts of cell-associated and non-associated mycoplasma. They appear to be less for the primary cells than for any of the transformed cell types. Thus, the apparently high resistance of the primary cells could also, in part, be related to their less heavy contamination. For this interpretation the possibility must be excluded that the orcein staining technique, as used, may not demonstrate the mycoplasma equally well for the different cell types. Such differences, however, cannot help explain the relative resistance to infection of the mycoplasma-modified cells, since similar amounts of cell-associated mycoplasma were observed for these cells and FL cells after comparable infection. These amounts can be so excessive (Fig. 10) that mechanical interference with normal cell functions must be considered as an explanation for the changes in growth and cultural behavior.

The present results may also apply to the practical problems in tissue culture contamination. Mycoplasma infections of cell lines are often detected only when special techniques for observation or isolation are employed, and the frequent lack of obvious signs of infection could well be the result of inapparent, previous infections which might have been cured by the application of various routinely used antibiotics. Such infections can have selected for, or changed, the original mycoplasma-sensitive cell population to a population of relatively resistant cells which do not respond to reinfection with easily observed cell destruction or morphological changes. Due to regular and frequent tests, we have good evidence that our cultures of FL cells, during many years of continuous cultivation, have never been accidentally contaminated with mycoplasma. Further studies along this line should include other cell types and newly developed cell lines to determine if similar resistance can be induced, also with other types of common mycoplasma contaminants.

Summary. The population doubling time for lines of FL human amnion cells infected with mycoplasma fermentans (strain HT) was increased (30 hr) compared to uninfected FL cells (17.5 hr). Infected FL cell lines from which mycoplasma was eliminated, had a doubling time of 21 hr and showed an increased resistance to reinfection, even several years after the mycoplasma elimination. Although the amounts of cell-associated mycoplasma were similar to those of infected FL cells, cytopathic effects and cell destruction were much less pronounced after reinfection of such lines. The amounts of cell-associated, as well as free mycoplasma, were reduced in cultures of primary amnion cells as compared to the transformed cells. Primary amnion cells were highly resistant to mycoplasma infection as judged by the number of glass-attached cells and lack of cell destruction. The theoretical and practical implications of these observations are discussed.

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