Polymyxin B sulfate therefore, appears not to be acting by the same mechanism described by Lorand and Jacobsen(4) for certain amine and carbonylamide inhibitors namely, the incorporation of the inhibitor into the fibrin molecule as a result of the action of FSF. In view of the effect of polymyxin B sulfate described here, it may be worthwhile to caution clinicians administering this drug parenterally to be alert for abnormalities in blood coagulation in their patients.

Several other antibiotics were studied for *in vivo*, anti-FSF activity in dogs. The antibiotics, the dose administered intravenously and the number of animals treated are as follows: neomycin sulfate ("Micifradin", The Upjohn Co.), 15 mg/kg—2 dogs, 50 mg/kg—1 dog, vancomycin hydrochloride ("Vancocin", Eli Lilly and Co.), 12 mg/kg—3 dogs, kanamycin sulfate ("Kantrex", Bristol Labs.), 7.5 mg/kg—3 dogs and colistimethate sodium ("Coly-Mycin", Warner Chilcott Labs.), 5 mg/kg—3 dogs. No evidence of anti-FSF activity could be demonstrated with any of these antibiotics.

Summary. Of several antibiotics investigated, polymyxin B sulfate showed anti-FSF activity following intravenous administration to dogs. Peak activity was found at approximately one hour and the effect persisted through the third hour post drug. *In vitro* experiments, in which cysteine was found to antagonize the anti-FSF activity of polymyxin B sulfate, suggest that the antibiotic interferes with fibrin stabilization by a reversible inactivation of FSF.

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Morphological and Quantitative Aspects of Mycoplasma-Human Cell Relationships.*† (32110)

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The method for direct microscopical demonstration of mycoplasma (Pleuropneumonia-like organisms, PPLO) in cultured cells(1), has been used extensively in diagnostic work to test for contamination of tissue and cell cultures; it has proven rapid and reliable. The technique, involving hypotonic treatment of the cells, air drying, and staining with orcein, has permitted detailed observations of the characteristic morphology of the mycoplasma-mammalian cell association. Such observations can be applied quantitatively to an evaluation of the amounts, or to actual counts, of mycoplasma attached to the mammalian cells. The present report is based upon an analysis of the correlation between mammalian cell-associated mycoplasma and mycoplasma present in the fluid phase of cell cultures under various experimental conditions.

Materials and methods. Cells. FL human amnion cells cultured serially in LY Medium (2), with 20% human serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were transferred with trypsin, 0.05%, and for experiments seeded and infected in 1 ml medium in "Demuth cups" (Demuth Glass Works, Division, Brockway Glass Co.) with

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inserted round cover slips; incubated in an atmosphere of 5% CO_2 in atmospheric air.

Mycoplasma. Strain HT(3), cultured either in Bye broth(4) with 15% human serum, or in cultures of FL cells. This mycoplasma is sensitive to Aureomycin(5) and kanamycin (6), but resistant to penicillin and streptomycin.

Method for direct microscopical demonstration of mycoplasma in cell cultures. Reagents: Sodium citrate solution, 0.6%; Carnoy's fixative; orcein stain; and procedure as previously reported(1). Slides were examined under the microscope, using phase optics, at magnifications from 400× to 3200×.

Mycoplasma titration on agar plates. The concentration of colony-forming units (CFU) was determined by inoculating proper dilutions of test samples into 35 mm plastic Petri dishes containing Bye agar(4) with 15% human serum. The plates were incubated at 37°C in an atmosphere of 5% CO_2 in nitrogen. Colony numbers were determined microscopically either by total plate counts or by extrapolation of counts of randomly selected representative areas.

Counting of FL cells. For seeding of cultures cell numbers were determined on trypsinized cell suspensions from hemocytometer counts. The actual number of cells in each experimental sample was determined under a dissection microscope from the orcein-stained preparations, either from counts of cells on the total slide or by extrapolation of counts of randomly selected representative areas.

Gradation of the amount of FL cell-associated mycoplasma. A culture of FL human amnion cells infected with mycoplasma will after some time, when prepared according to the direct demonstration method, show the individual microorganisms associated with the cell surface, located primarily at the cell borders, and attached to the peripheral parts of the cytoplasm. The number of cell-associated mycoplasma vary with the mycoplasma inoculum and with time of incubation from few individual microorganisms to heavy confluent masses covering the cells on all available sides and apparently most concentrated near and in the intercellular spaces. In order to study quantitatively the relation between mycoplasma and the mammalian cells over this whole range of amounts of infection, it was necessary to make two types of measurements: a) for light amounts of FL cell-associated mycoplasma actual counts of the microorganisms could easily be obtained at high microscopical magnification; b) for experiments involving heavy infection it was necessary to define certain progressive grades of mycoplasma-FL cell association. Fig. 1 and 2 demonstrate the 4 degrees of mycoplasma association adopted in these studies and defined as follows:

+++: Heavy mycoplasma association with all FL cells, even of densely populated cultures. The number of mycoplasma may

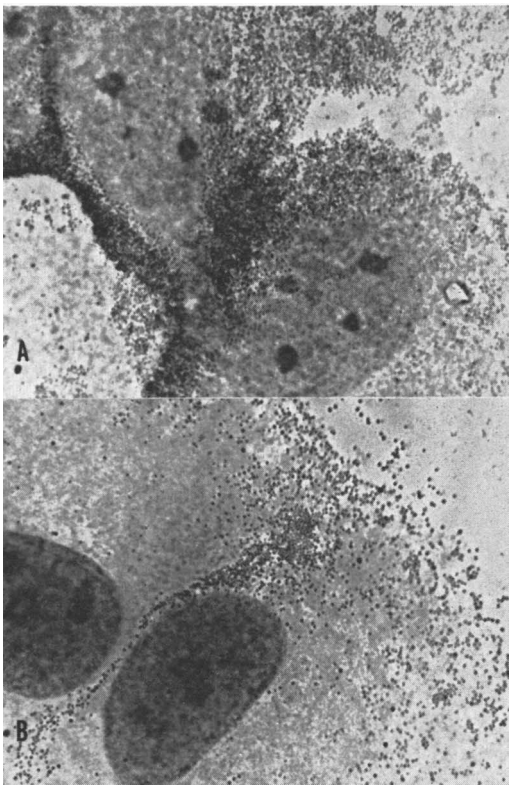


FIG. 1. and 2. Mycoplasma-infected FL human amnion cells after preparation and staining with orcein according to the described technique; observed under phase microscopy at magnification: 833×.

FIG. 1, A and B. +++ grade mycoplasma-mammalian cell association. A. Density of the microorganisms too high for counts; B. More than 500 microorganisms per cell.

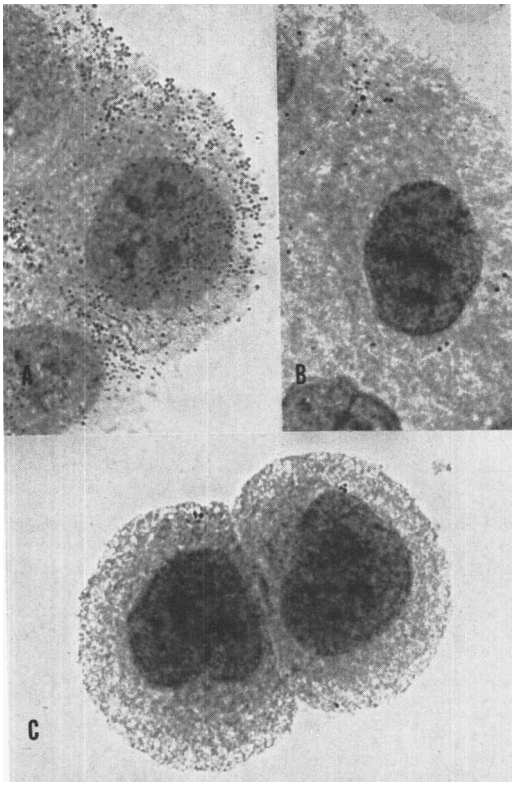


FIG. 2, A, B, and C. These pictures demonstrate grades ++, +, and (+) respectively of cell-associated mycoplasma. Number of mycoplasma per cell can easily be counted.

become so high that individual microorganisms cannot be distinguished (Fig. 1, A); in light dense cultures there are more than 500 mycoplasma per cell (Fig. 1, B).

++: Medium heavy mycoplasma association with all or most cells; the distribution of mycoplasma is non-uniform in dense cultures, uniform in cultures of low cell density; there are between 60 and 500 mycoplasma per cell in light dense cultures (Fig. 2, A).

+: There is a light degree of mycoplasma association with only some of the cells of dense cultures and with all cells of low density cultures. There are 20 to 60 mycoplasma per cell in light dense cultures (Fig. 2, B).

(+): Only some cells, even of light cultures, show a few (less than 20) associated mycoplasma (Fig. 2, C).

Results. 1. *FL cell-associated mycoplasma in relation to mycoplasma inoculum and culture fluid titer.* In order to study this relationship 4 groups of cups were seeded with the same number of FL cells and, 24 hours later, were inoculated with 10-fold serial dilutions of a mycoplasma suspension. Samples of the culture supernatants were collected for titration on agar plates, and the cell sheets were exposed to hypotonic treatment, air drying and staining with orcein at 6, 24, 48, and 72 hours after mycoplasma inoculation. The number of cells per slide and the amount of FL cell-associated mycoplasma were determined by microscopic observation. Each sample was prepared in duplicate.

The results are shown in Fig. 3. After an inoculum of $10^{5.2}$ CFU/ml, maximum titer in the culture supernatant (10^8 CFU/ml) was reached within 24 hours; with an inoculum of $10^{2.2}$ CFU/ml, 72 hours were required to obtain the maximum titer. The data show that +++ degree of FL cell-associated mycoplasma was obtained when the culture fluid titers reached above $10^{7.5}$ CFU/ml. Between 10^7 and $10^{7.5}$ CFU/ml the grade ++ for cell-associated mycoplasma was observed; between $10^{6.5}$ and 10^7 CFU/ml the amount of cell-associated mycoplasma was estimated at + and, in the interval between 10^6 and $10^{6.5}$ CFU/ml, cell-associated mycoplasma was (+). It is indicated from the data that the amount of cell-associated mycoplasma within the experimental period, is independent of the time of incubation, but is strongly related to the number of CFU/ml in the culture supernatant.

Although all cultures were seeded with the same number of cells, the number of cells per slide varied in this experiment between 10,000 and 200,000; this variation is related both to cell division during the experimental period and to cell destructive changes effected by the mycoplasma infection.

2. *Effects of variation of the number of FL cells.* As demonstrated in Table I, the degree of FL cell-associated mycoplasma varied considerably with the number of FL cells per culture. In this experiment, 24 cultures were seeded with FL cells at 6 different densities, 4 cultures per cell density, and all received, on the following day, the same mycoplasma inoculum (4.8×10^6 CFU). Cultures were prepared for direct mycoplasma observation at

TABLE I. Degree of FL Cell-Associated Mycoplasma in Cultures of Various Cell Densities at 6, 12, 18, and 24 Hr After Mycoplasma Inoculation (4.8×10^6 CFU/Cup).

Hours	No. of FL cells/cup	FL cell-associated mycoplasma
6	1,750 – 6,500	(+)
	≧ 25,500	0
12	2,400 – 4,700	+
	10,900 –105,000	(+)
18	≦3,800	+++
	6,900 – 64,800	++
	≧ 99,100	+
	4,400 – 15,200	+++
24	24,500 –101,000	++
	≧135,000	+

6, 12, 18, and 24 hours, the number of cells per slide was counted, and amount of FL cell-associated mycoplasma determined. The summarized data in Table I show clearly that the degree of FL cell-associated mycoplasma for any period of time increases with decreasing numbers of FL cells. For the lowest numbers of cells studied, cell-associated mycoplasma could be observed in the earliest samples, *i.e.*, 6 hours after mycoplasma inoculation.

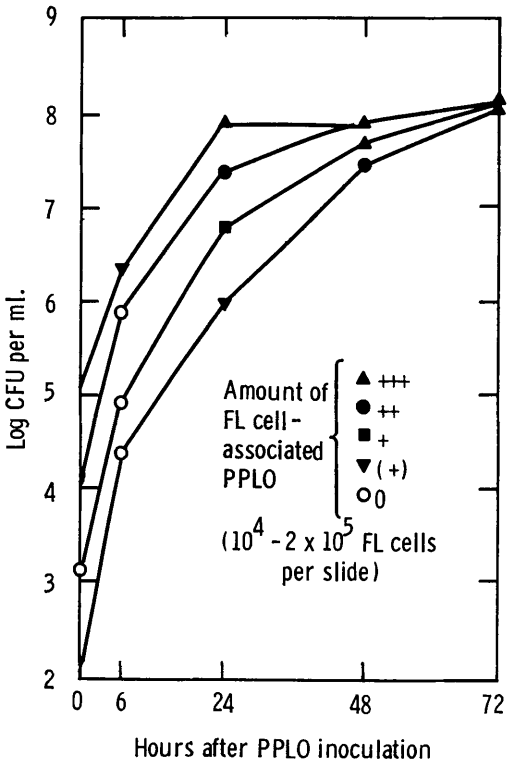


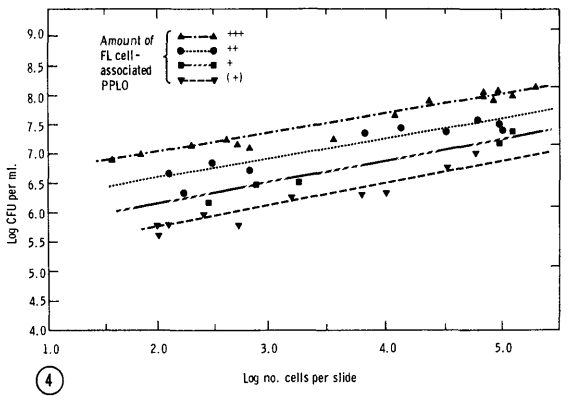
FIG 3. Amount of FL cell-associated mycoplasma (PPLO), expressed as degrees, related to number of mycoplasma colony-forming units (CFU) per ml in the cell culture supernatant after various periods of incubation. Cell seeding (high) constant; 4 mycoplasma inocula.

Results from a similarly designed experiment, performed with very low numbers of FL cells per culture and with sampling in the period from 2 to 12 hours after mycoplasma inoculation, are shown in Table II. The Table shows that FL cell-associated mycoplasma may be observed as early as 2 hours after inoculation of 3×10^5 CFU mycoplasma. The actual numbers of mycoplasma per FL cell are shown in the last column. The first number is the average for 20 cells; the range of numbers counted for the 20 cells is shown in the parenthesis. The logarithm of these numbers plotted against time form an essentially straight line.

TABLE II. Degree and Actual Numbers of FL Cell-Associated Mycoplasma in Cultures of Very Low Cell Densities at 2 to 12 Hr After Mycoplasma Inoculation (3.0×10^5 CFU/Cup).

Hours	No. of FL cells/cup	Amt of FL cell-associated mycoplasma	No. mycoplasma per FL cell
2	112–289	(+)	1.6 (0– 4)
4	87–238	(+)	3.8 (0– 6)
6	41–246	(+)	10.5 (4– 27)
8	82–273	+	54.7 (28–102)
10	30–363	++	70.7 (29–115)
12	199–584	++	395 (130–*ca.* 800)

Data from a number of different experiments, representing various inocula and times of infection with mycoplasma, are collected in Fig. 4, to illustrate the relationship between amount of FL cell-associated mycoplasma and concentration of CFU in the culture fluid over a great range of numbers of FL cells per culture. A straight line relationship is indicated in this system. It can be seen that maximum supernatant titers increase gradually as the number of cells increases. At lower cell numbers a certain degree of cell-associated mycoplasma correspond to lower mycoplasma titers in the culture fluid than at higher cell numbers. (When the cell number is decreased, from 10^5 to 10^2 cells per culture, the same degree of cell-associated mycoplasma is ob-



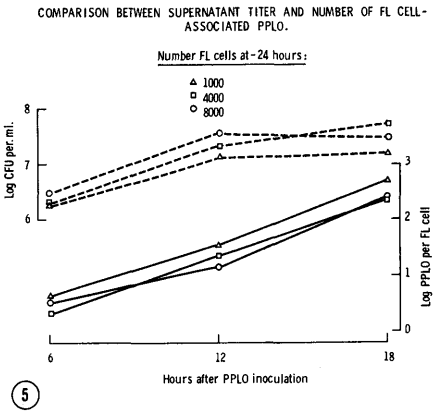


FIG. 4. Amount of FL cell-associated mycoplasma (PPLO), expressed as degrees, related to CFU per ml in the cell culture supernatant for a range of FL cell numbers. Data originating from different experiments include variations in mycoplasma inoculum and incubation period.

FIG. 5. Text shown on Figure.

served at a supernatant titer of approximately 10 times less.) It is obvious, therefore, that low numbers of FL cells are preferable when this method is being used in diagnostic work for mycoplasma testing.

3. *Comparison between mycoplasma titers in culture supernatant and FL cell-associated mycoplasma determined by actual counts.* FL cells were seeded at three different concentrations, 1000, 4000, and 8000 cells per cup. All cups received similar mycoplasma inoculum 24 hours after seeding. In Fig. 5 each point on the lower graphs represents the average of counts of mycoplasma on 20 FL cells; the 3 upper graphs show the corresponding titers of mycoplasma in the culture fluid. The data indicate that in the first 6-hour experimental period the rates of increase of mycoplasma in the culture fluid and of FL cell-associated mycoplasma were similar; the subsequent decrease in rate in the culture fluid during the next 6-hour period (unchanged rate for cell-associated mycoplasma) may indicate a "saturation level"(3) in the supernatant, most likely explained by nutritional exhaustion. These data also show that the number of CFU per ml in the supernatant at the high number of cells is higher than at lower cell numbers. The number of cell-associated mycoplasma per cell is higher at lower numbers of cells. It can be seen that the proportion of CFU per ml in the supernatant to cell-associated mycoplasma per cell increases with increasing number of cells. The relative amounts of mycoplasma in culture fluid and in association with the total cell population for each of the experimental situations can be calculated. Based on the number of cells seeded per culture 24 hours previous to mycoplasma inoculation, the ratio CFU per ml to number of cell-associated mycoplasma varies between 400:1 and 125:1 for 6- and 12-hour samples; for the 18-hour samples, this ratio is only between 63:1 and 16:1.

4. *Effects of variation of temperature.* A series of cup cultures were prepared similarly with the same low number of FL cells and subsequently inoculated with the same amount of mycoplasma. One-third of the cultures were incubated at 37°C, one-third at 30°C, and the remaining cultures were kept at 24°C. Samples were collected during the following 96 hours for determinations of culture supernatant titers and for direct observation of the degree of FL cell-associated mycoplasma. As shown in Fig. 6, +++ FL cell-associated mycoplasma was obtained at culture fluid titers of $10^{6.8}$ at 37°C, but more than 10^{8} CFU/ml in the fluid was required to obtain +++ at 30°C. The same pattern was observed for the other degrees of FL cell-mycoplasma association. Thus, (+) was observed corresponding to $10^{5.5}$ CFU/ml after incubation at 37°C; whereas at 30°C, 10^{6} CFU/ml was required. At 24°C, $10^{6.2}$ CFU/ml was required for a (+) association, and at this temperature only a few mycoplasma were associated with FL cells at any time

during the experiment, even as the mycoplasma titer in the supernatant approached $10^{6.8}$ CFU/ml.

Discussion. This investigation was aimed primarily at studying the function of the mammalian cells in respect to the quantitative aspects of mycoplasma propagation in cell cultures. The high fragility of mycoplasma excludes use of physical-chemical techniques, applicable to virus-host cell studies, for the determinations of amounts of cell-attached mycoplasma. Such determinations, which were necessary for this study, were possible by direct microscopical observations of hypotonic-treated and orcein-stained cell cultures. For numbers of mycoplasma not exceeding approximately 500 per cell, exact counts could be obtained; at heavier levels of infection the quantitative determination depended on defined grades. The data have shown reproducible relations between number of mammalian cells, mycoplasma inoculum, titer in the culture supernatant, and the amount of cell-associated mycoplasma pointing to the possibility of using systems as here reported, for example, to determine the activity of a sample of unknown titer. It has been strongly emphasized from the results that low numbers of FL cells are preferred when the direct microscopical demonstration method is used for diagnostic testing.

Although the HT and other strains of mycoplasma can propagate in certain routine tissue culture media without presence of cells (3), it has previously been shown that presence of cells results in increase of the amount of mycoplasma as titered in the culture supernatant(3); the present data have shown that supernatant titers increase with the number of cells per culture. It has been speculated that mammalian cells either provide the mycoplasma with certain nutrients absent from the medium or that they may inactivate substances that prevent growth of the mycoplasma(7); it can be added that better conditioning of the medium and, for example, pH adjustment may be accomplished by more mammalian cells present. The data presented in this report confirm not only that mycoplasma are located to a large extent in connection with the mammalian cells, on the cell surface and most likely inside the cells, but also show that mycoplasma may selectively favor the cell site, rather than the fluid culture phase, for their location of propagation since, for a given supernatant titer, more cell-associated mycoplasma is observed per cell at lower than at higher cell numbers (Fig. 4).

Previous observations of mycoplasma-infected FL cells under the electron microscope (8) have demonstrated an intimate relation between mycoplasma and mammalian cell surfaces. The borders of the infected mammalian cells were characteristically projected in the form of microvilli in the neighborhood of the microorganisms and the plasma membrane of the mycoplasma may become continuous with the plasma membrane of the mammalian cell at the tips or along the sides of the microvilli, suggesting attachment. In some cases attachment appeared to be in the non-villated regions of the cell border. The attachment was generally by direct apposition of plasma membranes, but often appeared to be through plasma membrane extensions. That cell attachment involves an active biological process on behalf of either cell or microorganism is also indicated from the data in the present report by the reduction in the proportion of microscopically observed cell-associated mycoplasma to free mycoplasma which occurs after temperature reduction (Fig. 6). It could be speculated that receptor sites for mycoplasma on the mammalian cell surface cease to function as temperatures are decreased below 37°C.

The data in Fig. 5 indicate that the number of cell-associated mycoplasma increases after the time when the number has reached maximum in the fluid phase. This suggests more favorable conditions (nutritional a.o.) for mycoplasma propagation in association with the mammalian cell. Along this line should be mentioned that in mycoplasma-infected mammalian cells exposed to hypotonic treatment and stained with orcein, it is a common finding to observe considerable cytoplasmic destruction in areas of the cytoplasm crowded with mycoplasma (Fig. 1A); cytoplasmic breakdown was also characteristic in electron microscopic studies(8,9). It is most likely that a considerable proportion of the myco-

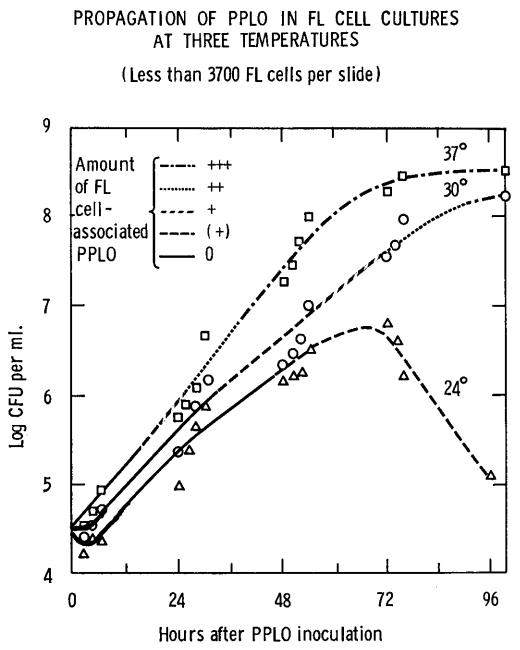


FIG. 6. Text shown on Figure.

plasma demonstrated in the culture fluid is derived from cell-associated replication. In spite of the high ratios between the number of mycoplasma in the fluid phase and the number of cell-associated mycoplasma, all our data point to the fact that mycoplasma in the cell culture propagates preferably in association with the mammalian cells and that the number of mycoplasma observed microscopically in connection with the cells is not an expression of random distribution.

In this report two types of expressions for mycoplasma amounts have been compared, colony-forming capacity on agar and visible morphological entities associated with the cells. The first measurement obviously relates to biological activity, since it indicates number of units with the capacity to divide; the entities observed as mammalian cell-associated, orcein-stained bodies on the cell surface or intracellularly do not *per se* prove viability from their morphological appearance under the microscope. However, since their increase with time is logarithmical and the rate of increase similar to rate of increase of colony-forming units in the fluid phase, as demonstrated in this report, we can conclude that the observed and counted cell-associated mycoplasma are also living organisms.

This report has demonstrated the possibility of very detailed quantitative, morphological observations of mycoplasma-human cell interrelationships. It has recently been shown that this association can lead to changes in the genetic composition(10) of the human cells, changes which are stable after elimination of mycoplasma from the cultures and which are also reflected in a reduced tumor-producing capacity of the cells (11). Obviously the quantitative systems reported here lend themselves to continued study of effects of environmental conditions, of antibiotics, of mixed infections, etc.

Summary. Microscopic observations of mycoplasma-infected FL human amnion cells exposed to hypotonic treatment, air drying, and staining with orcein according to the procedure previously reported, have yielded new information concerning the morphology and quantitative relationships between mycoplasma and mammalian cells. At lower levels of infection amounts of mycoplasma per cell can be determined by actual counts; for more heavily infected cultures according to defined degrees. There is a correlation, independent of the incubation period (within limits), between the quantity of cell-associated mycoplasma and mycoplasma titer (colony-forming units per ml) in the cell culture fluid. Maximum value of this titer increases gradually with the number of FL cells per culture. Increase of cell-associated mycoplasma with time is logarithmical. The amount per FL cell (other factors equal) increases with decreasing number of FL cells demonstrating that the mycoplasma compete for cell-association and are not randomly distributed in the culture. Cell-attached mycoplasma are also observed earliest in cultures containing low numbers of FL cells. Under such conditions mycoplasma (of strain HT) can be seen on cells within 2 hours after an inoculum of 3×10^5 colony-forming units. The proportion of free to cell-associated mycoplasma varied, in one experiment, between 400:1 and 16:1. At temperatures lower than 37°C (30°C, 24°C) this proportion increases.

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Immunohemagglutination Test for Rapid Detection and Assay of Rubella Antibodies.* (32111)

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Teratogenicity and widespread incidence have given rubella considerable contemporary importance. *In vitro,* rubella virus renders a number of mammalian cells resistant to superinfection with cytolytic viruses (8,15,17,19), produces recognizable cytopathology in others (6,18,19,21), but has lacked hemagglutinating or hemadsorbing activity(9). Apart from the fluorescent antibody technique described by Brown *et al*(1), slower, less sensitive complement fixation and neutralization tests have been the only procedures available for detection and assay of rubella antibodies(8,14,16, 20,21). This report describes the use of tanned chicken red blood cells coated with rubella virus for qualitative and quantitative determination of rubella antibodies in animal and human sera.

Materials and methods. Tissue culture. Cultures of LLC-MK_2 cells were grown and maintained in medium 199 containing 1% horse serum. BHK_{21} cells of the WI_2 clone, kindly supplied by Dr. S. A. Plotkin, were grown as monolayer cultures in Eagle's basal medium containing 10% calf serum, 10% tryptose phosphate broth (Difco) and antibiotics (penicillin, streptomycin and amphotericin B)(18). For cell maintenance and virus growth, the serum concentration was reduced to 2-5% and the tryptose phosphate broth omitted. For the growing of BHK_{21} cells in suspension, Eagle's basal medium, containing a double concentration of amino acids and vitamins, 10% calf serum, and 10% tryptose phosphate broth was used. For cell maintenance and virus growth in suspension culture, the double strength Eagle's basal medium was used with 5% fetal calf serum as the only supplement. All suspension cultures contained 3 ml of 4% methylcellulose (1500 cp) per 100 ml of medium. Monolayers of RK_{13} cells were grown and maintained in medium 199, 15% fetal calf serum and antibiotics(6). Uninfected tissue culture fluid (TCF) was passed in parallel with virus and used for controls.

To grow virus in monolayer cultures, cells were infected with undiluted seed virus, allowed one hour at room temperature for adsorption, then maintenance medium added and bottles reincubated at 35°C. Virus was grown in suspension cultures by infecting cell suspensions at high multiplicity with the viral inoculum, and the cultures agitated on a rotary shaker at approximately 300 rpm. Both virus and control TCF were harvested by

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