REV. PER. BIOL. 4 (1-2): 25-35, DIC. 1992

MYCOPLASMA DETECTION IN A MOUSE CELL LINE AND POSSIBLE CONTAMINATION SOURCE

WILLIAN CORNEJO M. * and LIBERTAD ALZAMORA G. **

Instituto de Medicina Tropical, Facultad de Medicina, UNMSM *
Instituto de Investigación en Ciencias Biológicas "Antonio Raimondi", Facultad de Ciencias Biológicas, UNMSM **

SUMARIO

Se aisló y detectó una cepa de micoplasma contaminante de la línea celular NS-1. Para el aislamiento primario, se empleó Agar PPLO suplementado con suero equino o humano y extracto de levadura. Como métodos indirectos de detección se emplearon la línea celular indicadora Vero y el marcador fluorescente de DNA Hoeschst 33258. El microplasma fue aislado de NS-1, así como también de Vero. Sin embargo sólo con las células Vero fue posible obtener colonias de micoplasma con la típica apariencia de "huevo frito". Cultivos celulares de Vero, mantenidos de 2-5 días en el medio de cultivo conteniendo los sueros por probar fueron marcados con Hoechst. Las células Vero mostraron características y discretas zonas fluorescentes en el citoplasma y el núcleo. El microorganismo produjo efecto citopático sobre las células indicadoras, alcanzando su mayor desarrollo luego de la tercera o cuarta semana de cultivo. No se observó desprendimiento de la monocapa celular. El método de la marcación fluorescente de DNA parece ser el medio de elección para la detección de micopasma, debido a que resultó ser má rápido, eficiente y fácil de ejecutar.

SUMMARY

The detection of cintaminating mycoplasma in a NS-1 myeloma cell line was studied comparing three methods The probable source of mycoplasma contamination were some batches of commercial sera. Fetal Bovine Sera (FBS), mouse myeloma NS-1 cells and Vero cells (African green monkey) were cultured on PPLO medium. The other methods involved the use of an indicator cell culture system (Vero cells) and the DNA-fluorochrome staining technique. The bacteriological procedure for the isolation of mycoplasma was successful with NS-1 and Vero cells, but not with FBS. However, mycroplasma colonies with typical "fried egg" appearance were only observed with Vero cells. Moreover, the number of colonies isolated could be appreciated only after 18 days of growth. Vero tested, showed cytopathic effect (CPE). Initially, dark grasules appeared in the cytoplasm of the cells. At the 5th or 7th day, cell membranes showed small finger-like projections and vacuolization. By the 3rd or 4th week, the CPE was more pronunced. Monolayers of Vero cells were also grown on coverslips for 2 to 5 days and were stained with Hoechts DNA fluorescent stain. The cells showed discrete zones of fluorescence in the cytoplasm and nuclei. The fluorescent spots were time-dependent Hoechst technique appears to be the method of choice, since it is more efficient, less time consuming and simpler.

INTRODUCTION

Since the appearance of the first reports of cell cultures contaminated by Pleuropneumonia-Like Organisms (PPLO), a term previously used to describe Mycoplasma (Robinson, LB., et al., 1956), there has been published a series of well do cumented literature (Collier, L. H., 1957; Hearn, H. J., 1959; Rothblat, G. E., 1959; Pollock, M. E., 1960; Hayflick, L., 1960· Carski, T. R., 1961). However, since the microorganisms are not easily detectable, Mycoplasmas have been ignored in expe rimental procedures. In most cases as the result of contamination there are no changes either in growth patterns or in cell morphology (Rothblat, G. H., 1960).

In recent years it has become evident that Mycoplasmas can produce numerous effects on cell cultures in terms of antigenicity of the cell membrane, macromolecular synthesis, stability of genetic material, sensitivity to virus and to pharmaco logical drugs and other parameters (Powelson, D. M. 1961; Hakala, M. T., 1963; Randall, C. C., 1965; Fogh, J., 1965; Rouse, H. C., 1963; Somerson, N. L., 1965; Gafford, L. G., 1969; Singer, S. H., 1969; Kenny, G. E., 1963; Afshar, A., 1967; Butler, M., 1964). This evidence has encouraged many researchers to look for sensitive methods for the demonstration of these microorganisms (Edward, D. G., 1947; Chanock, R. M., 1962; Zgorniak-Nowosielska. I., 1967; House, W., 1967; Fogh, J., 1964; Barile, M. F., 1962; 1963; Shedden, W. I., 1966; Todaro, G. J., 1971; Chen, T. T., 1971 Russell, W. C., 1975).

The purpose of this communication is to describe three methods for the detection of Mycoplasma in NS-1 myeloma cell line which was contaminated in the laboratory during culture maintenance. This contamination was responsible for endless screening of many commercial sera as these were suspected to be the source of Mycoplasma.

MATERIAL AND METHODS

Material tested for Mycoplasma Contamination

Mouse myeloma NS-1 cells were kindly supplied by Jaime Castillo of Universidad Nacional Agraria, Lima. This myeloma cell line was recovered from frozen state and the cells were grown during a week at Dr. Castillo's laboratory before we received the cells at our Institute. It was demonstrated that the cell line was at that time free of contaminating agents.

After receiving the cell line in our laboratory, it was maintained in continuous passages for 10 days. By the time of the second subculture the NS-1 cells showed vacuolization, and aggregated cell masses could be observed. These characteritics were more evident after serial sub-passages and they suggested the presence of a cytopathic agent. The growth medium used was Dulbecco's Modification of Eagle's Medium (DMEM, Gibco) supplemented with 2 mM glutamine (Sigma), 10% inactivated Fetal Bovine Sedum (FBS, Gibco) and 40 μg/ml gentamicin. This is referred to as "10% FBS-DMEM". Of. all the reagents, FBS had been recently purchased and thus was considered the probable source of contamination for the NS-1 cell culture.

Vero cells (African green monkey), kindly supplied by the Cellular Biology and Virology Laboratory, Universidad Peruana Cayetano Heredia, Lima, were used as an indicator cell culture to detect the possible mycoplasma infection in the NS-1 cell line. They also were maintained in complete DMEM, except for a PPLO-free serum batch. For Mycoplasma detection, Vero cell lines were cultured using com-

plete DMEM supplemented with sera from different batches. No antibiotic was used in Vero cells growth medium.

Mycoplasma Isolation by Microbiological Method

Solid medium consisted of PPLO Agar (Difco), 20% non-inactivated horse serum and 2.5% yeatst extract (Oxoid). PPLO broth was prepared from Beef heart Inrusión (De Lustig, E. S. 1981) and contained 20% non-inactivated horse serum and 2.5% yeast extract. Later, horse serum was successfully replaced in both media by human serum, extracted from one of us.

Six ml of the FBS, suspected of contamination, were inoculated directly on 50 ml of PPLO (De Lustig, E. S., 1981; Bira, B. R., 1981). Broth cultures were incubated aerobically at 37°C, in humidity, either in the presence or in absence of 5% carbon dioxide. 0.2 ml aliquots from each broth culture were sub-cultured on PPLO Agar petri dishes at days 6 and 15 (Bira, B. R., 1981; McGarrity, G. J., 1985). They were incubated as previously stated and maintained during 10-15 days before being discarded.
Samples of a Vero Cell suspension of 5, 18 and 21 days of growth were inoculated on PPLO Agar petri dishes. Parental Vero cells, maintained in a mycoplasma-free growth medium were plated on PPLO agar as controls.

NS-1 cells of 6 days of growth, were inoculated on PPLO Agar plates. Both Vero and NS-1 cells were incubated under the same conditions as described for broth cultures.

Indicator Cell Culture for Mycoplasma Contamination

Vero cell culture was selected as indicator system (McGarrity, G. J., 1985) for the detection of mycoplasma from contaminated serum. The Vero cells had been grown in plastic T-flasks, in Eagle's Minimum Essential Medium (MEM) containing 10% inactivated FBS (56°C for 30 minutes) and 40 μg/ml gentamicin. The cells were adapted in DMEM without antibiotics for two weeks before using them as our indicator system. Cells were dispersed for passage using 0.25% Trypsin (Difco) in Hank's Balanced Salt Solution without calcium nor magnesium salts (BSS), after rinsing them once with BSS. After detachment from the plastic flasks, the cells were suspended in complete DMEM. Then, aliquots were transferred into T-flasks. Cells were transferred ai 5-day intervals to fresh medium and were ready for experiments after the 4th passage.

Cultures to be assayed for PPLO detection from contaminated serum were stabilized for 24 hours in plastic flasks in 10% FBS-DMEM medium.which was then discarded and replaced with similar 1% FBS containing medium Cells were subcultured during one month and showed a marked cytopathic effect (CPE) in the last passages. At different intervals of growth, cell suspensions were also cultured on PPLO Agar (See above).

Control Vero cell cultured never showed CPE and Mycoplasma was not isolated from these control cultures.

DNA Fluorochrome Staining Technique

A suspension of the indicator Vero cell culture was added to, and grown on a glass coverslip placed inside plastic petri dishes. They were then incubated at 37°C in a 5% CO_2 incubator. The dishes were previously fed with complete DMEM containing the serum suspected of being contaminated. Petri dishes with mycopasma-free control growth medium were also used. Indicator Vero cells were allowed to grow up to 50-80% confluence

only, because it is difficul to interpret results in totally confluent monolayers. The cells were ready 2-5 days after incubation. After growth, the cells were fixed and stained with DNA fluorochrome Hoechst 33258 stain (Flow Lab) following instructions from manufacturer.

In each test, 3T-6 mouse fibroblast cells infected with *Mycoplasma hyorhinis* (DBS-1050) and non-infected 3T-6 mouse fibroblast cells, were included as positive and negative controls, respectively.

The converslips to be assayed were mounted on glass slides containing mounting fluid. 1 or 2 drops of immersion oil were added to the coverslip's surface and then examined by fluorescence microscopy under an AO Flourestar microscope.

RESULTS

Attempts to Isolate Mycoplasma from FBS

Beef Heart Infusion broth bottles inoculated with FBS were incubated for 2 weeks at most. After 6 days' incubation, one group of them was centrifuged and the sediment was inoculated on solid medium. No PPLO organisms were observed in these cultures during the time of incubation. PPLO Agar dishes inoculated with the sediments collected from broth medium after two weeks of incubation, as described, were also found free of mycoplasma.

Mycoplasma Isolation from Cell Cultures

At the third sub-culture of NS-1 cells, suspensions were inoculated on PPLO Agar. Eleven to fifteen days after inoculation, the agar plates showed the presence of opaque microareas distinguishable under microscopic examination. These colonies were scarce, of circular shape and granular appearance.

Vero cell cultures were sub-cultured through 7 passages wihin 30 days. At the first sub-culture, it was not possible to isolate Mycoplasma. However, mycoplasma colonies with typical "fried egg" appearance were observed at 18 and 21 days of growth (Figures 1-3). The Mycoplasma strain could be maintained by transferring from one agar plate to another, using the agar-block technique.

All cell cultures yielded Mycoplasma with apparently no difference regarding the presence or absence of CO_2.

Cytopathic Effects in Vero Cell Cultures

The Vero cells were sub-cultured by a 2:1 split ratio at 3-4 day intervals, at which time a confluent monolayer developed. Cultures were maintained without cytopathic effect (CPE) whenever mycoplasma-free growth medium was used (Figure 4). However, this changed when the cells were maintained in DMEM containing the suspected FBS. The CPE, although early in time, was light. Initially, dark granules appeared in the cytoplasm of the cells. At the 5th or 7th day, cell membranes showed small finger- like projections and vacuolization. A few days later, the cytoplasm of adjacent cells seemed coalescence and 2, 3 or 4 nuclei could be seen inside one cell. Although CPE progressed slowly, it was more conspicuous by the 3rd or 4rd week, when cells rounded up or elongated, acquiring a spindle-like shape or forms with cut-ends (Figures 5-6). During this study, Vero cells never detached spontaneously from the plastic surface.

Mycoplasma Detection in Indicator Vero Cells using Hoechst Stain

Vero cells which had grown over coverslips in petri dishes were maintained for 2 to 5 days in DMEM containing the sera to be tested. Cells were stained with

Hoechst 33258 DNA-fluorescent stain, and examined by fluorescent microscopy at 1000X in immersion oil.

Vero cells showed characteristic discrete zones of green fluorescence in the cytoplasm, and around the cellular and nuclear membranes. When cells were grown for 2 days, only a few fluorescent spots were observed; the number of these spots increased in 5-days cultures. Throughout the assay, non-infected cells always gave negative cytoplasmic fluorescence.

DISCUSSION

Detection of contaminating mycoplasma in a myeloma cell culture was studied comparing three methods currently used for the demonstration of the presence of Pleuropneumonia-like Organisms.

The use of a standard assay procedure for the isolation of Mycoplasma demonstroted its presence both in NS-1 Myoloma and Vero cell lines. However, in the former case, the number of visibles colonies in agar was very small compared with the number of colonies isolated from Vero cell cultures. In addition, mycoplasma colonies with the typical 'fried egg" appearance were only obtained from Vero cell culture inoculations. These differences may be attributed to the larger number of passages (McGarrity, G. J., 1985) for Vero cells before inoculations. Furthermore, for Mycoplasma isolation, Vero cells had to grow for more than 5 days in DMEM medium containing the FBS which was being tested, as it was observed that a 3-5 days culture was not enough (data not shown).

In the present study, the CO_2 requirement seemed not to play an important role for mycoplasma isolation, because organisms could be isolated both in the presence or obsence of carbon dioxide. Most important, it was possible to isolate Mycoplasma under aerobic conditions although many studies have reported more isolates with anaerobic incubations (McGarrity, G. J., 1979). Direct FBS inoculation of broth cultures resulted in failure of Mycoplasma isolation on PPLO Agar dishes, despite waiting up to two weeks of broth incubation before inoculating the samples into PPLO Agar dishes. This failure to isolate Mycoplasma from contaminated FBS is explained by the fact that it was not possible to inoculate large amounts ot FBS, as in the method reported by Barile and Kern (Bira B. R., 1981; Barile, M. F., 1978) where a minimal of 25 ml inoculation of serum was used. We could only inoculate 6 ml of test serum because of the scarcity of available broth medium. It is possible that if mycoplasma is present in low concentrations, e. g., one or two organisms per liter, these may not be detected by direct culture.

Vero cell culure was used as indicator cells for early detection of Mycoplasma likely to be present in FBS. Small numbers of Mycoplasma can reach high concentrations within a few days upon inoculation in to the indicator cell cultures (Barile, M. F., 1981). This was evident when comparing the growth of Vero cells in test medium with the growth of the same cells in PPLO-free control medium. Vere cell cultures maintained in test medium showed slight CPE at the beginning, wish progressed until complete destruction of cell monolayer. Initial effect consisted of cellullar granularity and then prominent vacuolization. In contrast, routine examination of conrol cultures never revealed the presence of CPE.

The effect of Mycoplasma on cell morphology varies according the Myco plasma species —or even strain— cultured cell line and type of medium employed (McGarrity, G. J., 1985; Stanbridge, E. 1971). Girardi et al (1965), using primary cultures of African green monkey kidney (GMK) cells, found that cells appear gra

nular, shrunken and refractile. Also, alte red cells readily detached from the glass. Similar results were reported by Hummeler et al (1965) working with GMK, Hela and other tissue cultures using Mycoplasma isolated from bening human tumors. They found that the lesions were comparable in all cell types tested, although the timing of the CPE presentation and the severity differed: the effect was earlier (3-4 days) and more pronounced in primary GMK, secondary chicken embryo and Hela cell cultures, than in the other cell lines.

A Vero cell monolayer of 50-80% confluence was fixed and stained with the DNA fluorescent Hoechst stain ,and examined under a fluorescent microscope. The Vero cells were previously allowed to grow for 2-5 days in the medium containing the serum to be tested. Positive cell cultures showed nuclei surrounded by singular small dots of fluorescence, whose numbers increased by sampling older cultures. By combining the fluorescent DNA stain with the indicator cell technique, Del Giudice and Hoops (1978) reported a total of 9 false-positive and 13 false-negatives for 2,297 specimens examined. Although occasionally nuclear fragments distinguished from Mycoplasma by their large size and brigther fluorescence (flow Lab).

Comparing the three methods used, staining with Hoechst DNA stain was the less time-consuming procedure, easiest to perform and did not require many reagents. Although other pro-caryotic non-mycoplasma organisms can also be detected with this technique, instead of this being a disadvantage, it constitutes a quick screening procedure to detect contaminants. So far, it has been observed that mycoplasma, bacteria, yeasts and other procaryotic cells may be easily demonstrated by this method. The use of the indicator cell cultures together with the florescent DNA stain provides better standardization and allows appropriate positive and negative controls to be included in each assay. Some false-negative results have been reported (McGarrity. G. J. 1979), due to non cyto-adsorbing mycoplasmas, but its incidence was low. Like several other detection methods, the indicator cell cultures have been useful in some occasions. But, it is critical to select the appropriate indicator cell line in order to assure that the method is really capable of detecting various mycoplasma species that could be present in the tested specimen. The use of indicator cells alone, although it allowed us to know if the FBS was contaminated with mycoplasma or not, was not as sensible as the DNA staining method. This last procedure does not provide a diagnosis, since some virus may produce similar CPE that the one originated from mycoplasma contamination.

On the other hand, Mycoplasma can be detected by the direct method. Although this method defects the presence of most mycoplasma contaminants, there are some reports of misleading negative results. Besides, artifacts production may constitute a problem for inexperienced personnel, since it can interfere with mycoplasma colonies recognition.

All evidence obtained suggested that commercial bovine serum was the source of mycoplasma contamination of the NS-1 myeloma cell line. Supplier promotes sera quarantine, and mycoplasma and virus contamination screening.

ACKNOWLEDGEMENS

We wish to thanks Dr. Cristina Escajadillo for her critical revision of the manuscript.

We also thank Mr. Néstor Huaylas for all the photographic work.

Our grateful acknowledgement to Ana Gil for supplying us with the Hoecht Staining Kit.

REFERENCES

AFSHAR, A. (1967). The growth of M. *bovigenitalium* in cell cultures, J. Gen. Microbiol. 47, 103-110.

BARILE, M. F., MALIZIA, W. F. and RIGGS, D. B. (1962). Incidence and detection of pleuropneumonia like organisms in cell cultures by fluorescent antibody and cultural procedures. J. Bacteriol. 84, 130-136.

BARILE, M.F. and SCHIMKED, R.T. (1963). A rapid chemical method for detecting PPLO contamination of tissue cell cultures. Proc. Soc. Exp. Biol. Med. 114, 696-679.

BARILE, M. F., HOPPS, H. E. and GRABOWKI, M. M. (1978). Incidence and sources of mycoplasma contamination: a brief review, p. 35-45. In McGarrity, G. J., Murphy, D. G. and Nichols, W. W. (ed), Mycoplasma enfection of cell cultures.

BARILE, M. F. (1981). Mycoplasma infections of cell cultures. Isr. J. Med. Sci. 17, 555-562.

BIRA, B. R. and FORRESTER. F. T. (1981). Basic Laboratory Techniques in cell culture, U. S. Department of Health and Human Sources. Centers for Disease Control.

BUTLER, M. and LEACH, R. H. (1964). A mycoplasma which induces acidity and cytopathic effect in tissue culture. J. Gen. Microbiol. 34, 285-294.

CARSKI, T. R. and SHEPARD, C.C. (1961). Pleuropneumonia-like (mycoplasma) infections of tissue culture, J. Bacteriol. 81, 626-635.

COLLIER, L. H. (1957). Contamination of stock lines of human carcinoma cells by pleuropneumonia-like organisms. Nature 180, 757-758.

CHANOCK, R. M., HAYFLICK, L. and BARILE, M. F. (1962). Growth on artificial medium of an agent associated with atypical pneumonia and its identification as a PPLO. Proc. Natl. Acad. Sci. U.S. 48, 41-49.

CHEN, T. R. (1971). In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. Exp. Cell. Res. 104, 255-262.

DEL GIUDICE. R. A. and HOPPS, H. E. (1978). Microbiological methods of mycoplasma infection of cell cultures, p. 57-69. In: McGarrity, G. J., Murphy, D. G. and Nichols, W. W. (ed), Mycoplasma infection of cell cultures.

DE LUSTIG, E. S. and NEBEL, A. E. (1981). Cultivo de tejidos. Un manual práctico.

EDWARD, D. G. (1947). A selective medium for pleuropneumonia-like organisms. J. Gen. Microb. 1,238-243.

FOGH, J. and FOGH, H. (1964). A method for direct demonstration of pleuropneumonia-like organisms in culture cells. Proc. Soc. Exp. Biol. Med. 117, 899 901.

FOGH, J. and FOGH, H. (1965). Chromosome changes in PPLO-infected FL human ammnion cells. Proc. Soc. Exp. Biol. Med. 119, 233-238.

FLOW Laboratories, Instructions sheet.

GAFFORRD, L. G., SINCLAIR, F. and RANDALL, C. C. (1969). Growth cycle of fowl pox virus and change in plaque morphology and cytopathology by contaminating mycoplasma. Virology 37, 464-472.

GIRARDI, A. J., HAMPARIAN, V. V., SOMERSON, N. L. and HAYFLICK, L. (1965). Mycoplasma isolates from primary cell cultures and human diploid cell strains. Proc. Soc. Exp. Biol. Med. 120, 760-771.

HAYFLICK, L. and STINEBRING, W. R. (1960). Intracellular growth of pleuropneumonia-like organisms (PPLO) in tissue culture and in OVO. Ann. N. Y. Acad. Sci. 79, 433-449.

HAKALA, M. T., HOLLAND, J. F. and HOROSZEWICZ, J. S. (1963). Change in pyrimidine deoxyribonucleoside metabolism in cell culture caused by mycoplasma (PPLO) contamination. Biochem, Biophys. Res commun. 11, 466-471.

HEARN, H. J., OFFICER, J. E., ELSNER, V. and CROWN, A. (1959). Detection, elimination and prevention of contamination of cell cultures with pleuropneumonia-like organisms. J. Bacteriol. 78, 575-58.

HOUSE, W. and WADDELL, A. (1967). Detection of mycoplasma in cell cultures. J. Pathol. Bacteriol. 93, 125-132.

HUMMELER, K., ARMSTRONG, D. and TOMASSINI, N. (1965). Cytopathogenic mycoplasmas associated with

two human tumors. II. Morphological aspects J. Bacteriol. 90, 511-516.

KENNY, G. E. and POLLOCK, M. E. (1963). Mammalian cell cultures contaminated with pleuropneumonia-like organisms. I. Effect of pleuropneumonia-like organisms on growth of established cell strains. J. Infec. Dis. 112, 7-16.

McGARRITY, G. J., SARAMA, J. and VANAMAN, V. (1979). Factors influencing microbiological detection of mycoplasmas in cell cultures. In Vitro 15, 73-81.

McGARRITY, G. J., SARAMA, J. and VANAMAN, V. (1985). Cell culture techniques. ASM News 51, 170-183.

POLLOCK, M. E., KENNY, G. E. and SYVERTON, J. T. (1960). Isolation and elimination of pleuropneumonia-like organisms from mammalian cell cultures. Proc. Soc. Exp. Bio. Med. 105, 10-15.

POWELSON, D. M. (1961). Metabolism of animal cells infected with mycoplasma. J. Bacteriol. 82, 288-297.

RANDALL, C. C., GALFORD, L. G., GENTRY, G. A. and LAWSON, L. A. (1965). Lability of host-cell DNA in growing cell cultures due to mycoplasma. Science 149, 1098-1099.

ROBINSON, L. B., WICHELBRAUSEN, R. H. and RAZINAN; B. (1956). Contamination of human cell cultures by pleuropneumonia organisms. Science 124, 1147.

ROTHBLAT, G. E. and MORTON, H. E. (1959). Detection and possible source of contaminating pleuropneumonia-like organisms (PPLO) in cultures of tissue cells. Proc. Soc. Exp. Biol. Med. 100, 87-90.

ROTHBLAT, G. H. (1960). PPLO contamination in tissue cultures. Ann. N. Y. Acad. Sci. 79, 430-432.

ROUSE, H. C., BONIFAS, V. H. and SCHLESINGER, R. W. (1963). Dependence of adenovirus replication on arginine and inhibition of plaque formation by pleuropneumonia-like organisms. Virology 20, 357-365.

RUSSEL, W. C. NEWMAN, C. and WILLIAMSON, D. H. (1975). A simple cytochemical technique for demonstration of DNA in cells infected with mycoplasmas and viruses. Nature (London) 253,462.

SHEDDEN, W. I. and COLE B. C. (1966). Rapid method for demonstrating intracellular pleuropneumonia-like organisms in a strain of hamster kidney cells (BHK21 Cl, 13). Nature, 210, 868.

SINGER, S. H., BARILE, M. F. and KIRSCHSTEIN, R. L. (1969). Enhanced virus yields and decreased interferon production in mycoplasma-infected hamster cells. Proc. Soc. Exp. Biol. Med. 131, 1129-1134.

SINGER S. H., FITZGERALD, E. A., BARILE, M. F. and KIRSCHSTEIN, R. L. (1970). Effect of mycoplasma on vaccinia virus growth: requirement for arginine. PProc. Soc. Exp. Biol. Med. 133, 1439-1442.

STANBRIDGE, E. (1971). Mycoplasma and cell cultures. Bacteriol. Rev. 35, 206-227.

SOMERSON, N. L. and COOK, M. K. (1965). Suppression of rous sarcoma virus growth in tissue cultures by *Mycoplasma orale*. J. Bacteriol. 90, 534-540.

TODARO, G. J., AARONSON, S. A. and RANDS, E. (1971). Rapid detection of mycoplasma infected cell cultures. Exp. Cell. Res. 65, 256-257.

ZGORNIAK-NOWOSIELSKA, I., SEDWICK, W. D., HUMMELER, K. and KOPROWSKI, H. (1967). New assay procedure for separation of mycoplasmas from virus pools and tissue culture systems. J. Virol. 1, 1227-1237.

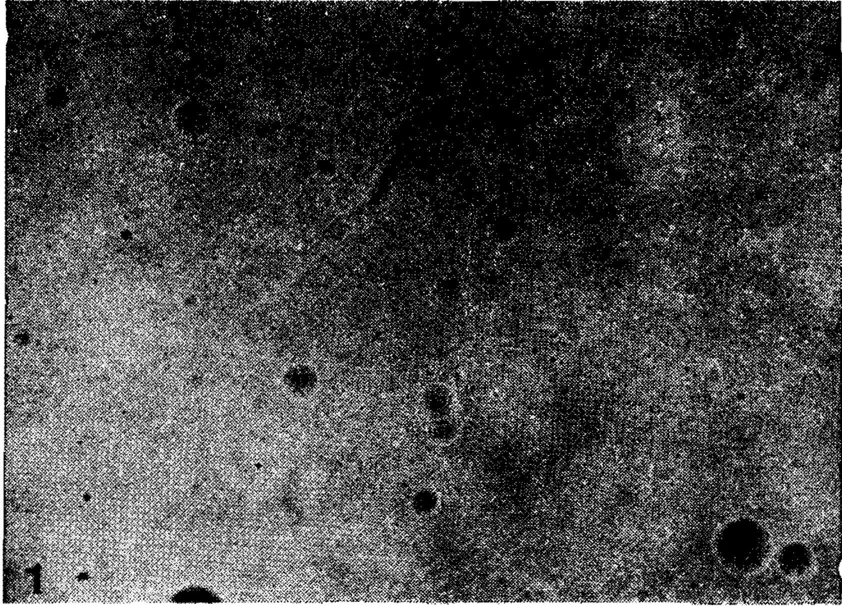

Fig. 1: Mycoplasma colonies en PPLP medium. (10X).

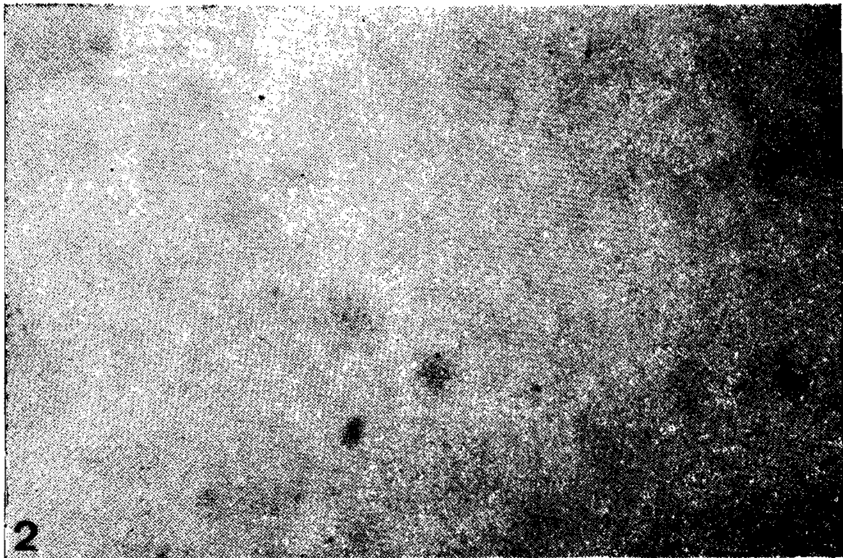

Fig. 2: Mycoplasma colonies with typical "Fried egg". (10X)

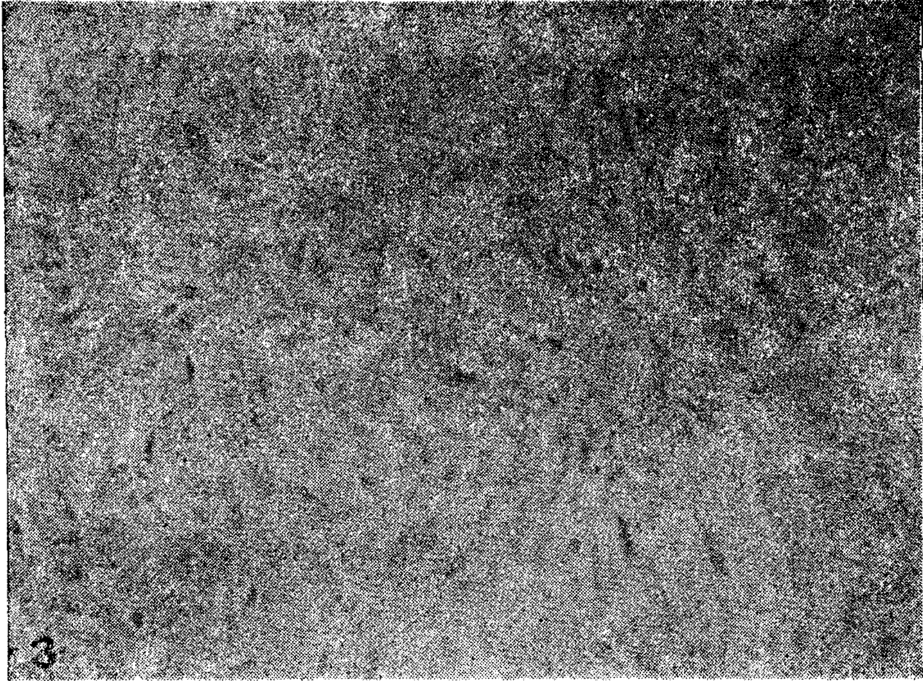

Fig. 3: Monolayer of VERO colla whitout Cytopatric effwet (CPE) (40X)

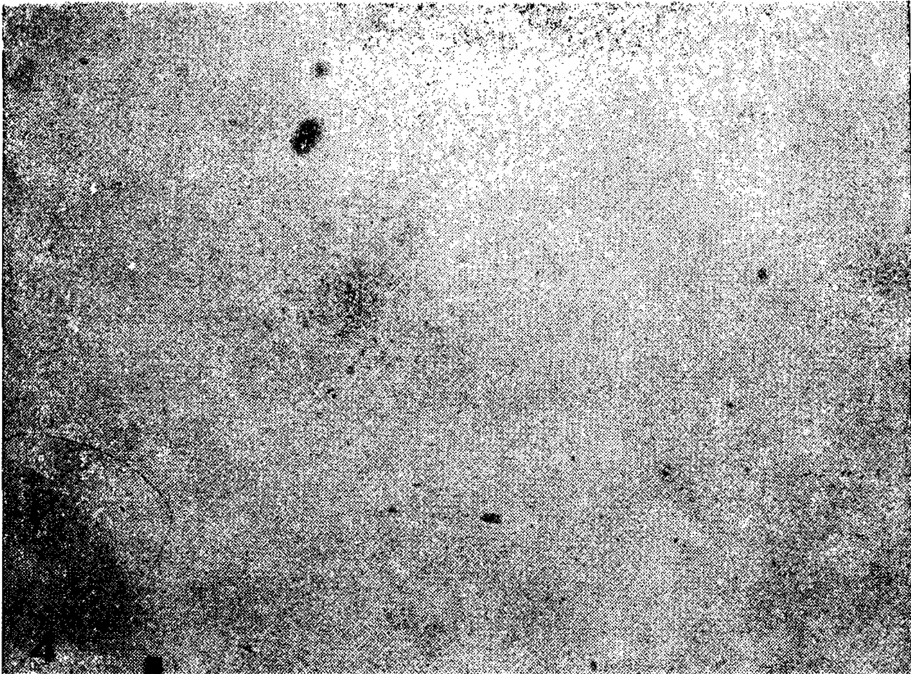

Fig. 4: Mycoplasma colonies (40X) en PPLO medium

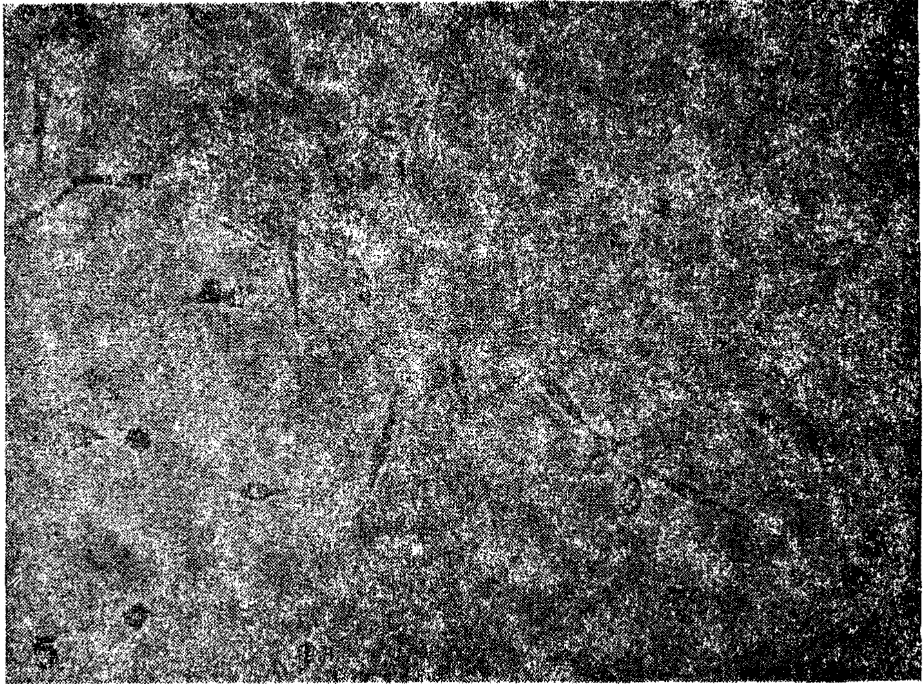

Fig. 5: Cytophathic effect (CPE) of Mycoplasma en monolayer of Vero cells. (40X)

Fig. 6: Cytophatic offect (CPE) of Mycoplasma en monolayer of Vero cells before 4rd week. (40X)