JOURNAL OF BACTERIOLOGY, Aug., 1965
Vol. 90, No. 2

Cytopathogenic Mycoplasmas Associated with Two Human Tumors

I. Isolation and Biological Aspects

D. ARMSTRONG, G. HENLE, N. L. SOMERSON, AND L. HAYFLICK

Virus Laboratories at the Children's Hospital of Philadelphia, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, National Institute of Allergy and Infectious Diseases, U.S. Public Health Service, Bethesda, Maryland, and Wistar Institute, Philadelphia, Pennsylvania

Received for publication 5 April 1965

ABSTRACT

ARMSTRONG, D. (The Children's Hospital of Philadelphia, Philadelphia, Pa.), G. HENLE, N. L. SOMERSON, AND L. HAYFLICK. Cytopathogenic mycoplasmas associated with two human tumors. I. Isolation and biological aspects. J. Bacteriol. **90**:418-424. 1965.—Mycoplasmas were isolated from cell cultures of two benign human tumors. The first isolate contained two mycoplasmas, one a well-known human species (*Mycoplasma hominis* type I) and frequent tissue culture contaminant, and the other a recently reported new type. The second isolate was a mycoplasma of the newly described type. The mycoplasmas could be reisolated, after one or more passages through tissue culture, from extracts of the original tumor tissue. The relationship of the organisms to the neoplasms remains obscure. Both isolates produce cytopathic effect (CPE) and acidification of medium in a variety of tissue cultures. The CPE may be diminished, but not abolished, by increasing concentrations of arginine in the tissue culture media. Infection of various tissue cultures with the mycoplasmas did not result in interference to superinfection with vesicular stomatitis virus.

Mycoplasmas [pleuropneumonia-like organisms (PPLO)] have recently been isolated by use of tissue culture or directly on agar in studies of human tumor material (Grace and Horoszewicz, *personal communication;* Girardi et al., 1965b; Murphy, Furtado, and Plata, 1965; Schmidt, Barile, and McGuinniss, 1965; Hayflick and Koprowski, 1965; Sabin, *personal communication;* Somerson and Lewis, *unpublished data*). In addition, particles resembling mycoplasmas have been observed by electron microscopy in association with both human and animal neoplasms (Dmochowski, 1964). This study concerns cytopathogenic agents which have been isolated from cultures of two benign human tumors. These isolates proved to be mycoplasmas. They were reisolated from the original tumors after passage through various types of tissue cultures which in themselves were demonstrably free of contaminating agents.

Although contamination of the specimens could have occurred upon removal of the tumors or during the initial processing of the tissue, the possibility that these agents were associated with the tumors in vivo deserves attention. The mycoplasmas reported here are of interest, since they cause a cytopathic effect (CPE) in tissue cultures with acidification of the medium and belong to a new group of previously unclassified agents (Butler and Leach, 1964; Girardi et al., *in preparation;* Hayflick, *in press*).

MATERIALS AND METHODS

Processing of tumors. Parts of the tumor specimens removed at surgery were washed three times with Hanks' balanced salt solution (BSS) which contained 100 units of penicillin and 0.1 mg of streptomycin sulfate per ml. When larger tumor specimens were obtained, small pieces were separated and stored at −20 C. The remaining portion was minced and treated with 0.25% trypsin. Tissue residue left after trypsinization was also stored at −20 C.

The trypsinized cells were washed and suspended in Eagle's basal medium (BME) containing 10% inactivated calf serum (56 C for 30 min) and penicillin and streptomycin in concentrations mentioned above. Subsequently, the calf serum concentration was increased to either 20 or 40%. Furthermore, finely minced tissue or trypsinized cells were placed on cultures of the human diploid cell strain WI-38 (Hayflick, 1965*a*) and handled as indicated below.

Cultivation of tumor CS-1. Tumor CS-1, a hemangioma of the knee of a 1-week-old male, was trypsinized for 2 hr at room temperature. Tubes

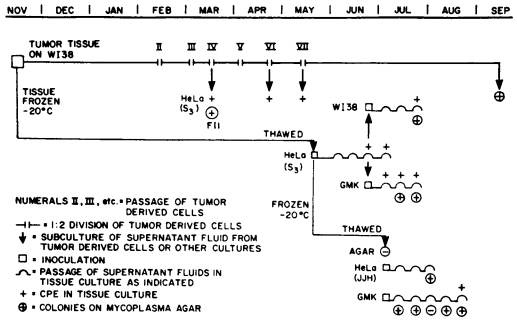

FIG. 1. *Schematic representation of cell culture of tumor CS-1 and subcultures on various tissue cultures and mycoplasma agar.*

planted with 8×10^5 cells per milliliter showed growth of fibroblasts which slowly degenerated after 1 month. With the hope that a normal human cell strain would promote growth of the tumor cells, tubes containing WI-38 cells were inoculated with the trypsinized tumor cells or minced tumor tissue. In these cultures, morphologically different types of fibroblasts were apparent by the 10th day, i.e., shorter cells with a more granular cytoplasm. These tumor-derived cells continued to grow and seemed to be the only type of cell present after the 90th day of incubation. Control cultures of WI-38 cells maintained on the same biweekly feeding schedule had degenerated by this time. The tumor-derived cultures were subcultivated in a 1:2 ratio and monolayers of fibroblasts with a granular cytoplasm developed by the 14th day. With use of BME with 20% inactivated calf serum for both growth and maintenance, further passages were made at 10- to 21-day intervals (Fig. 1). By the sixth subculture, growth had slowed, and after the seventh passage at 175 days, no further cell proliferation was observed. Ultimately, the cell number decreased until only a few very granular fibroblasts remained at 350 days.

Cultivation of tumor RF-1. Tumor RF-1, a posterior pharyngeal fibroma from a 13-year-old female, was trypsinized for 16 hr at 4 C. Cells seeded directly in tubes did not grow. In mixed cell cultures, with use of trypsinized tumor cells or minced tumor tissue with WI-38 cells, slow growth of shorter, more oval, and granular fibroblasts occurred. By the 44th day, confluent monolayers of the tumor-derived cells had developed. Control tubes of WI-38 cells had degenerated by this time. Tubes containing cells from the tumor were trypsinized and subcultured at 7- to 18-day intervals. The growth medium consisted of BME with 40% inactivated calf serum, and maintenance medium contained 10 or 20% inactivated calf serum. Cells in the ninth passage at 162 days grew slowly, and the 10th subculture resulted in no growth. A few very granular fibroblasts remained at 338 days (Fig. 2).

Cell cultures and media. For various experiments, the following cells were mainly employed: (i) the S_3 and JJH lines of HeLa cells; (ii) primary African green monkey kidney (GMK) cells obtained from Flow Laboratories, Rockville, Md; and (iii) human diploid cells, strain WI-38. Uninoculated cultures were tested at frequent intervals and always in parallel with inoculated cultures for the presence of mycoplasmas. The stock cultures and controls were always found to be free from mycoplasmas.

Other heteroploid cell lines and primary cultures used occasionally are listed in Table 1. The human KB, rabbit kidney RK-13, and the hamster sarcoma cells are lines carried at the Virus Laboratories of the Children's Hospital of Philadelphia. The primary human embryonic kidney (HEK) and primary human amnion cells were purchased from Flow Laboratories, Inc., Rockville, Md. The primary guinea pig embryonic kidney cultures were obtained from Microbiological Associates, Inc., Bethesda, Md.

All of the cell cultures were maintained on BME with 2% inactivated calf serum (56 C for 30 min), penicillin, and streptomycin. For some experiments, L-arginine as free base (Nutritional Biochemicals Corp., Cleveland, Ohio) was added to BME in up to 5.5 times the usual amount (17.4 mg/1,000 ml). In other experiments, medium 199 (arginine content, 70.0 mg/liter) or medium L-15 (Leibovitz, 1963; arginine content, 500 mg per liter) were used in comparison with BME.

Isolation of mycoplasmas. The agents described in this report were identified as mycoplasmas by use of the medium previously described and used for the isolation and identification of the Eaton agent as a mycoplasma (Chanock, Hayflick, and Barile, 1962). A complete description of this medium formulation is presented elsewhere (Hayflick 1965*b*). Supernatant fluids from degenerating tissue cultures inoculated with material from the tumors were found to contain mycoplasmas when placed on agar. The mycoplasma isolated from tumor CS-1 was designated F-11 and that from tumor RF-1 designated F-12.

Mycoplasma identification. The source of prototype human mycoplasma strains and the prepara-

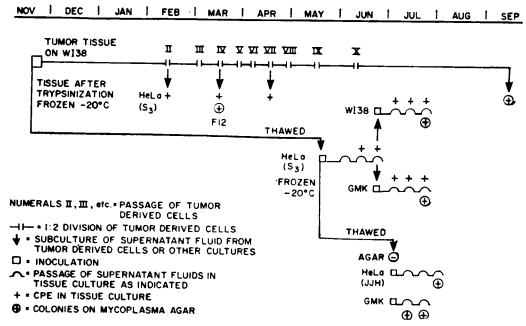

FIG. 2. *Schematic representation of cell culture of tumor RF-1 subcultures on various tissue cultures and mycoplasma agar.*

TABLE 1. *Tissue cultures in which F-11 and F-12 caused CPE and increased acidity*

Origin	Culture
Human	HeLa[a]
	KB
	WI-38[b]
	Primary human embryonic kidney[c]
	Primary human amnion[c]
Monkey	Primary green monkey kidney[d]
Rabbit	Rabbit kidney line RK-13
Guinea pig	Primary guinea pig embryonic kidney
Hamster	Hamster sarcoma line HT55 (Carr-Zilber Rous sarcoma virus-induced)
	Hamster sarcoma line 2HT (polyoma virus-induced)
Mouse	Mouse L(MCN) cell line[e]
Chicken	Second passage chick-embryo fibroblasts

[a] No evidence of interference to challenge with VSV after infection with F-11 or F-12, 10^3 $TCID_{50}$ or more, after 8 and 11 days.
[b] No evidence after 1, 3, 5, and 11 days.
[c] No evidence after 11 days.
[d] No evidence after 8, 11, and 14 days.
[e] No evidence after 7 days.

tion of antisera to these strains and to unclassified isolates has been described in detail elsewhere (Taylor-Robinson et al., 1963). Rabbits were immunized with antigens prepared from mycoplasmas that had been grown in mycoplasma medium previously described (Hayflick, 1965*b*) and then subcultured for at least four passages in rabbit infusion broth. Passage in the rabbit infusion medium was used to eliminate nonspecific reactions to broth medium constituents.

The mycoplasmas isolated from supernatant fluids of tissue cultures were subcultured on mycoplasma agar and in broth medium. Samples of the mycoplasma broth culture were sealed in ampules and stored at −70 C. To grow large quantities of organisms for preparation of complement-fixation (CF) antigens, the mycoplasmas were subcultured at 3- to 6-day intervals. After several serial passages, a CF antigen was prepared from organisms grown in the rabbit infusion mycoplasma broth medium and concentrated 10-fold by centrifugation at 18,000 rev/min for 45 min in a no. 20 head of a model L Spinco untracentrifuge. Antigens and sera were standardized by block titrations, and four units of antigen were used. Anticomplementary activity of the antigens, when present, was reduced by the following procedure: 9 parts of antigen and 1 part of guinea pig complement were incubated at 37 C for 1 hr. The mixture was then heated at 56 C for 30 min, and serial dilutions were tested in the CF test against known antisera to establish the appropriate dilution to be used. Rabbit antisera were heated at 56 C for 30 min. The tests were performed by the micro-complement-fixation procedure described by Sever (1962). Mycoplasma CF antigens from F-11 and F-12 were tested against 4 units of rabbit antisera prepared against six serotypes of human mycoplasmas: *Mycoplasma hominis* type 1, strain V2785; *M. hominis* type 2, strain Campo; *M. pneumoniae*, strain FH; *M. fermentans*, strain G; *M. salivarium*, strain Buccal; and *M. orale*, strain CH19299.

The growth-inhibition technique was that described by Clyde (1964) and performed as detailed by Taylor-Robinson et al. (1964).

Hemolysin production. The production of hemolysin by mycoplasma colonies growing on mycoplasma agar was tested by a technique described elsewhere (Somerson et al., 1965).

Sugar utilization. A sample (0.1 ml) of a broth suspension of mycoplasmas isolated from F-11 or F-12 was added to 10 ml of mycoplasma broth medium containing 1% glucose and 0.002% phenol red. These cultures and uninoculated broth medium were incubated at 36 C. *M. pneumoniae* and *M. salivarium* were used as positive and negative controls, respectively. A color change was indicative of acid production and was interpreted as sugar fermentation. To determine whether the organisms had grown, samples were removed from the inoculated broth cultures and grown on mycoplasma agar.

RESULTS

Isolation of cytopathogenic mycoplasmas from tumor CS-1 and RF-1 cell cultures. During the growth and maintenance of mixed WI-38 cultures with cells of tumors CS-1 or RF-1, the granular appearance of the cells suggested the presence of a cytopathic agent. At the third subculture of the CS-1 cells, supernatant fluids were inoculated into HeLa and GMK cultures. CPE was observed in HeLa cultures within 5 days. At the first subculture of RF-1 cells, supernatant fluids produced CPE after 4 days in HeLa cells. In WI-38 cells, CPE appeared after 14 days of the second passage. The agents isolated in HeLa or WI-38 cultures from both the hemangioma,

CS-1, and the fibroma, RF-1, could be serially passed in various cell cultures (Table 1). Coded specimens were tested for mycoplasmas, and isolates were obtained from both the CS-1 and RS-1 cell cultures. They were also grown from HeLa, GMK, and WI-38 tubes inoculated with supernatant fluids from CS-1 and RF-1 cultures. Typical mycoplasma colonies were observed on mycoplasma agar incubated for 4 to 7 days at 37 C. Uninoculated control cultures of HeLa, GMK, and human diploid cells did not yield mycoplasma colonies.

The mycoplasma strains F-11 and F-12 were subcultured through 10 passages on mycoplasma agar. After the 10th passage, colonies were removed from the agar surface and inoculated into WI-38 cultures. CPE appeared within 4 days, along with acidification of medium, and was indistinguishable from that produced by subculture of CS-1 and RF-1 supernatant fluids. The CPE and acidification have been transmitted through seven serial subpassages in WI-38 cells.

Stored frozen pieces of the tumor CS-1 and trypsinized residue from both tumor CS-1 and RF-1 were thawed, and 20% suspensions were made in phosphate-buffered saline solution (PBS) containing penicillin and streptomycin. HeLa cells inoculated with these suspensions showed CPE, and supernatant fluids were then inoculated into cultures of GMK and WI-38 cells. From these cell cultures, mycoplasmas F-11 and F-12 were again isolated. CPE and increased acidity appeared on serial passages in all three types of cultures. Mycoplasmas were not isolated from parallel, uninoculated control cultures of the three cell types. Reisolation from the original tumor tissue was again undertaken, and this time direct culture on mycoplasma agar was attempted with the twice-frozen and thawed tumor materials. Mycoplasmas could not be isolated directly from the original tumor suspensions or trypsinized residue. However, the GMK cultures inoculated with the two tumor specimens yielded mycoplasmas on agar, whereas the uninoculated control cultures again did not. Corresponding results were obtained with inoculated and control HeLa cultures. The mycoplasmas were reisolated also from frozen trypsinized residue of CS-1 tumor tissue by the same procedures described above.

Cytopathic and other effects of the isolates in cell cultures. The F-11 and F-12 mycoplasmas produced a similar CPE. The lesions were comparable in all types of cell cultures tested (Table 1), although they appeared earlier (3 to 4 days) and were more pronounced in primary GMK, secondary chick embryo, and HeLa cell cultures than in the others listed in Table 1. The cell membranes became indistinct, and the cytoplasm of adjacent cells seemed to coalesce. Initially, dark granules appeared at the periphery of the cells and around the nuclear membrane. At about the 7th day, granularity was replaced by prominent vacuolization. Finally, cells rounded up and detached from the glass surface. Details of these changes and the fine structure of the agents are described in the accompanying paper (Hummeler, Armstrong, and Tomassini, 1965).

Acidification of medium appeared within 3 to 4 days after inoculation of relatively large doses of the mycoplasmas or after refeeding of the infected cultures. After inoculation of progressively smaller doses of the mycoplasmas, the medium became acid in the absence of detectable CPE or the cultures showed neither acidification nor CPE, yet the F-11 and F-12 mycoplasmas could be subcultured on mycoplasma agar.

Effect of various media on the CPE. The CPE in primary GMK cultures was moderately delayed and decreased, but by no means abolished, by increasing the L-arginine concentration up to 5.5-fold in BME. Daily refeeding of the cultures also diminished CPE when compared with those refed on the usual biweekly schedule. With medium 199 or L-15, which contained up to 28 times the concentration of L-arginine found in BME, the CPE in primary GMK and HeLa cultures was undiminished after infection with F-11 and slightly diminished after infection with F-12.

Challenge experiments for detection of interference. The cell cultures indicated in Table 1 were inoculated with varying concentrations of F-11 and F-12 (from 10^3 to 10^7 colony-forming units per milliliter). After incubation periods ranging from 1 to 14 days, the cultures were superinfected with 100 $TCID_{50}$ of the Indiana strain of vesicular stomatitis virus (VSV). Tubes were examined daily after challenge, and sodium bicarbonate was added to any tubes showing increased acidity to attain the same *p*H as in the controls. There was no evidence that the mycoplasmas interfered with the production of CPE by VSV.

Attempts to relate the mycoplasmas to the patients. Mycoplasma could not be isolated from the throat of the patient from whom tumor RF-1 was removed. However, the culture was not attempted until 6 months after surgery. Both patients were bled shortly after surgery, and their sera were tested in serial dilutions for their capacity to neutralize 100 $TCID_{50}$ of F-11 and F-12 prior to inoculation of primary GMK cultures. Neither patient showed neutralizing antibodies to either mycoplasma at a serum dilution of 1:8.

Identification and classification of the myco-

plasmas. The mycoplasma isolate from cell cultures of CS-1 was subcultured for at least six passages before tests for CF reactivity, sugar utilization, and hemolysin production were performed. By this time there was no evidence of sugar fermentation or of β-hemolysin production. A CF antigen prepared from the mycoplasma strain F-11 reacted to a high level with rabbit antiserum to *M. hominis* type 1. Little or no CF reaction was observed when the antigen was tested with the five other rabbit antisera against mycoplasmas of human origin. These findings suggested that the mycoplasma strain F-11 was closely related to, if not identical with, *M. hominis* type 1. However, evidence of sugar utilization by the organisms had been obtained during the process of subculturing to prepare CF antigens. Furthermore, mycoplasma colonies were observed on mycoplasma agar medium incubated only 1 to 3 days. In contrast, isolates of *M. hominis* type 1 generally grow more slowly and do not ferment sugar. Acidification of tissue-culture media had been observed in cultures inoculated with mycoplasmas obtained from cell cultures of CS-1. Finally, mycoplasmas isolated from CS-1 and RS-1 both produced a similar CPE on reinoculation into tissue culture.

An early passage level of the mycoplasma F-11 isolate was grown in mycoplasma broth to which rabbit antiserum to *M. hominis* type 1 (at 10% final concentration) had been added. After five serial subcultures in the presence of antiserum, the surviving organisms were grown on mycoplasma agar medium without *M. hominis* type 1 rabbit antiserum. This "selected" culture produced acid in a glucose-enriched mycoplasma broth and gave β-hemolytic plaques when colonies growing on mycoplasma agar were overlaid with guinea pig red blood cells. Thus, the mycoplasma strain F-11 appears to be a mixture of at least two mycoplasmas: (i) organisms indistinguishable from *M. hominis* type 1 isolates, and (ii) organisms serologically distinct from the presently recognized human mycoplasmas. This second type grows rapidly, ferments sugar, and produces a β-hemolysin as tested with guinea pig erythrocytes.

The mycoplasma strain F-12 isolated from the RF-1 inoculated cell cultures is, at present, indistinguishable from the "selected" sugar-fermenting organisms of mycoplasma strain F-11. A CF antigen prepared from mycoplasma strain F-12 did not react with antiserum to *M. hominis* type 1. Preliminary attempts to relate any of the established mycoplasma species with the sugar-fermenting group have failed.

DISCUSSION

The isolation of cytopathogenic mycoplasmas from two tumor specimens is open to various interpretations. One may consider that (i) the organisms were etiologically related to the neoplasms from which they were derived; (ii) they were present in the tumor tissues merely as passengers or secondary infecting agents; or (iii) they represent superficial contaminants introduced after removal of the tumors from the patients.

The frequent contamination of cell cultures used in virus research by mycoplasmas and the resultant hazards in evaluating data are well known (Rothblat, 1960; Hayflick and Chanock, *in press*; Rouse, Bonifas, and Schlesinger, 1963). The discovery of cytopathogenic mycoplasmas which cause lesions in cell cultures similar to those induced by viruses (Grace et al., 1963; Butler and Leach, 1964; Girardi et al., *in preparation*; Pollock, Treadwell, and Kenny, 1963) and the occasional isolation of such agents during the investigation of tumors (Grace and Horoszewicz, *personal communication*; Girardi et al., 1965*b*; Murphy et al., 1965; Sabin, *personal communication*) emphasizes further the need for caution in the interpretation of in vitro cellular responses.

Whether these cytopathic mycoplasmas play a role in the etiology of certain neoplasms or represent passengers in the tumor is presently unknown. The problem demands attention and reaffirms the necessity for culturing all new, presumably viral, isolates for mycoplasmas, as well as maintaining constant surveillance of tissue cultures for inapparent mycoplasma infections.

From the data presented, it is unlikely that F-11 and F-12 were contaminants picked up during the course of cultivation of the tumor cells. Although attempts at direct isolation of the mycoplasmas on agar from suspensions of the tumors have failed, the organisms were recovered after their apparent enrichment in cell cultures. Direct isolation was attempted, however, only after the tumor specimens had been frozen and thawed twice, and thus the agents might have been largely inactivated. The fact that the mycoplasmas were isolated repeatedly from cell cultures inoculated on separate occasions with tumor materials and not from uninoculated control cultures denotes that they were associated with the tumor specimens. The detection of a mycoplasma antigenically related to *M. hominis* type 1 which was found in addition to the cytopathogenic agent in F-11 cultures may possibly represent a late contaminant. It cannot be excluded that the tumor specimens became con-

taminated during surgical removal or during the initial processing in the laboratory. Such an interpretation is suggested since (i) the agents were recoverable only after introduction of tumor tissue into cell cultures and therefore were present presumably only in low concentrations in the original material, and (ii) the patients possessed no neutralizing antibody to the mycoplasmas. However, the two tumors were processed 17 days apart, and other tumors handled on the same days or during the interval did not yield mycoplasmas.

If the agents were carried in the tumor, they probably represent passengers etiologically unrelated to the neoplasm. Neither tumor production by mycoplasmas in vivo (Girardi, Larson, and Hilleman, 1965*a*), nor cell transformation in vitro has been recorded. Both the F-11 and F-12 isolates have been inoculated subcutaneously into newborn and weanling hamsters, but to date (8 months) no tumors have appeared. In addition, cellular transformation has not been observed in primary GMK or WI-38 cultures inoculated with these mycoplasmas and maintained for 3 months.

The acid-producing fraction of the F-11 and the F-12 isolates belongs serologically to a recently observed group of mycoplasma which can cause distinct CPE and acidification of medium in various cell cultures (Butler and Leach, 1964; Girardi et al., *in preparation*). Their natural habitat is unknown, but members of this group have been isolated from both benign and malignant cell cultures. The F-11 and F-12 agents differ from previously described cytopathogenic mycoplasmas (Pollock, Treadwell, and Kenny, 1963; Kraemer, 1964; Hayflick and Chanock, *in press*) in that they induce cellular changes marked by prominent vacuolization and acidification of the culture medium which are only slightly reduced by addition of excess L-arginine. The diminished CPE due to increased L-arginine concentration in BME may depend on a balance between L-arginine and other constituents of the medium rather than L-arginine content alone. Media 199 and L-15 contain enough total L-arginine to delay or diminish CPE if that same concentration were present in BME. However, cultures maintained on 199 and L-15 showed almost the same degree of CPE as those maintained on standard BME containing lower concentrations of L-arginine.

The possibility that these mycoplasmas cause resistance of cell cultures to superinfection with viruses was explored with VSV as the challenge virus. Cell cultures infected with F-11 or F-12 isolates showed delayed CPE on VSV challenge on developing increased acidity of the medium. However, acidity is known to decrease the effectiveness of VSV (Bergs et al., 1958) and no resistance to VSV was evident when the infected cultures were maintained at the same *p*H as the controls. The metabolic activities of mycoplasmas infecting tissue cultures have been observed previously to interfere with viral multiplication (Rouse et al., 1963), but resistance due to interferon production by tissue cells has not been demonstrated.

ACKNOWLEDGMENTS

We would like to acknowledge the cooperation of C. E. Koop, Chief of Surgery, and J. P. Hamilton, Chief Resident in Surgery at Children's Hospital of Philadelphia, Philadelphia, Pa., in obtaining CS-1, RS-1, and many other surgical tumor specimens. The valuable suggestions of A. J. Girardi of The Wistar Institute are also acknowledged. The technical assistance of Regine Erismann, Barbara E. Walls, and Nancy Pleibel aided this work immeasurably.

This investigation was supported by Public Health Service grant CA-04568 from the National Cancer Institute to the Children's Hospital of Philadelphia, and by Public Health Service research grant CA-04534 and contract PH-43-62-157 from the National Cancer Institute to the Wistar Institute.

D. A. is a Special Fellow (5-F3-CA-13,562-02) of the National Cancer Institute and L. H. is the recipient of Public Health Service Career Development Award 5-K3-CA-5938.

LITERATURE CITED

BERGS, V. V., G. HENLE, F. DEINHARDT, AND W. HENLE. 1958. Studies on persistent infections of tissue cultures. II. Nature of the resistance to vesicular stomatitis virus. J. Exptl. Med. **108**: 561–572.

BUTLER, M., AND R. H. LEACH. 1964. A mycoplasma which induces acidity and cytopathic effect in tissue culture. J. Gen. Microbiol. **34**: 285–294.

CHANOCK, R. M., L. HAYFLICK, AND M. F. BARILE. 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.

CLYDE, W. A., JR. 1964. Mycoplasma species identification based upon growth inhibition by specific antisera. J. Immunol. **92**:958–965.

DMOCHOWSKI, L. 1964. Viruses as related to cancer. Proc. Natl. Cancer Conf., 5th.

GIRARDI, A. J., V. M. LARSON, AND M. R. HILLEMAN. 1965*a*. Further tests in hamsters for oncogenic quality of ordinary viruses and mycoplasma with correlative review. Proc. Soc. Exptl. Biol. Med. **118**:173–179.

GIRARDI, A. J., L. HAYFLICK, A. M. LEWIS, AND N. L. SOMERSON. 1965*b*. Recovery of mycoplasms in the study of human leukemia and other malignancies. Nature **205**:188–189.

GRACE, J. T., JR., J. S. HOROSZEWICZ, T. B. STIM, AND E. A. MIRAND. 1963. Mycoplasma isolated in tissue culture. Clin. Res. **11**:209.

HAYFLICK, L. 1965*a*. The limited in vitro lifetime of human diploid cell strains. Exptl. Cell Res. **37**:614–636.

HAYFLICK, L. 1965*b*. Tissue cultures and mycoplasmas. Texas Rept. Biol. Med. **23** (Suppl. 1): 285–303.

HAYFLICK, L., AND H. KOPROWKSI. 1965. Direct agar isolation of mycoplasmas from human leukemic bone marrow. Nature **205**:713–714.

HUMMELER, K., D. ARMSTRONG, AND N. TOMASSINI. 1965. Cytopathogenic mycoplasmas associated with two human tumors. II. Morphological aspects. J. Bacteriol. **90**:511–516.

KRAEMER, P. M. 1964. Interaction of mycoplasma (PPLO) and murine lymphoma cell cultures: Prevention of cell lysis by arginine. Proc. Soc. Exptl. Biol. Med. **115**:206–212.

LEIBOVITZ, A. 1963. The growth and maintenance of tissue culture in free gas exchange with the atmosphere. Am. J. Hyg. **78**:173–180.

MURPHY, W. H., D. FURTADO, AND E. PLATA. 1965. Possible association between leukemia in children and virus-like agents. J. Am. Med. Assoc. **191**:110–115.

POLLOCK, M. E., P. E. TREADWELL, AND G. E. KENNY. 1963. Mammalian cell cultures contaminated with pleuropneumonia-like organisms. II. Effect of PPLO on cell morphology in established monolayer cultures. Exptl. Cell Res. **31**:321–328.

ROTHBLAT, G. H. 1960. PPLO contamination in tissue culture. Ann. N.Y. Acad. Sci., vol. 79, Article 10, p. 430–432.

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

SCHMIDT, P. J., M. F. BARILE, AND M. H. MCGUINNISS. 1965. Mycoplasma (pleuropneumonia-like organisms) and blood group I, associations with neoplastic disease. Nature **205**:371–372.

SEVER, J. L. 1962. Application of a micro technique to viral serological investigations. J. Immunol. **88**:320–329.

SOMERSON, N. L., R. H. PURCELL, D. TAYLOR-ROBINSON, AND R. M. CHANOCK. 1965. Hemolysin of *Mycoplasma pneumoniae*. J. Bacteriol. **89**:813–818.

TAYLOR-ROBINSON, D., N. L. SOMERSON, H. C. TURNER, AND R. M. CHANOCK. 1963. Serological relationships among human mycoplasmas as shown by complement-fixation and gel diffusion. J. Bacteriol. **85**:1261–1273.

TAYLOR-ROBINSON, D., J. CANCHOLA, H. FOX, AND R. M. CHANOCK. 1964. A newly identified oral mycoplasma (M. orale) and its relationships to other human mycoplasmas. Am. J. Hyg. **80**:135–148.