Mycoplasma (PPLO) Strains with Lytic Activity for Murine Lymphoma Cells *in vitro*.* (28052)

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Several years ago, contaminant agents capable of completely destroying cultures of the murine lymphoma cell line L5178Y were isolated from several virus pools. These agents were discovered while studying the viral spectrum of L5178Y cells and were derived from virus pools grown in tissue culture. No association could be shown between the presence of the contaminants and any particular virus, and passage of the agents in L5178Y cultures resulted in complete loss of the viruses in question. Similar agents, sharing the same lytic activity for L5178Y cells *in vitro* have since been isolated from many uninoculated tissue culture strains and lines. This paper reports results of work which indicates that these agents represent a distinct group of mycoplasma.

Materials and methods. The L5178Y cell originated from a lymphoblastic leukemia of a DBA/2 mouse. The origin and serial cultivation of these cells have been described by Fischer(1). Cells were grown in stationary tubes (they do not adhere to the glass) with silicone stoppers and cultivated in serial passage with "L-1" medium to which was added calf serum (5%), penicillin (100 units/ml), and streptomycin (100 μg/ml). L-1 medium is similar to that reported by Fischer, the only difference being that a defined amino acid mixture(2) is substituted for the casein hydrolyzate and amino acids of the earlier medium.

For demonstration of mycoplasma colonies on agar the medium devised for cultivation of the Eaton mycoplasma was used(3). This consists of commercial PPLO agar (Difco) supplemented with freshly prepared (or stored at −20°C) yeast extract (10%) and unheated horse serum (20%). Colonies on agar were tested for their ability to retain the Dienes stain(4) by direct application of dilute stain to the colonies.

Tissue cultures, other than L5178Y cells, were generally grown in a modified Eagle's Basal Medium(5) obtained from Microbiological Associates, Inc. with added calf serum (10%), penicillin (100 units/ml), and streptomycin (100 μg/ml).

The first 2 agents studied have been referred to as "B-7" and "12-3". B-7 was isolated from a polyoma virus pool received from Dr. E. A. Mirand, Roswell Park Memorial Inst., Buffalo, N. Y. 12-3 was isolated from a Coxsackie virus pool obtained from Dr. K. Hummeler, Children's Hospital, Philadelphia, Pa. In addition, over a hundred independent isolations were made from various diploid and heteroploid cell cultures, most of which came from the same laboratory within a 2-month period. Those derived from human fetal material represented 2nd to 45th passage cultures, similar to those described by Hayflick and Moorhead(6). Lytic mycoplasma were also isolated from serially propagated human diploid cells but not from primary human fetal cultures; both strains were purchased from the same commercial source. In addition, one of 5 HeLa cell sub-lines tested was positive for lytic mycoplasma; this culture was also contaminated with non-lytic mycoplasma.

Most of the studies reported here utilized the B-7 and 12-3 strains as well as 4 strains isolated from human diploid cell strains and referred to as L.M.1, L.M.2, L.M.3 and L.M.4.

Results. Growth of lytic mycoplasma in L5178Y cultures. The average generation time of L5178Y cells in control cultures varied from 12 to 20 hours, with exponential growth occurring from a few thousand cells per ml up to 1.6×10^6 cells per ml, at which point the pH reached about 6.8 and multipli-

* Investigation was supported in part by grants from the Division of General Medical Services, and from Am. Cancer Soc.

† Leukemia Society Scholar.

TABLE I. Time Sequence of Lytic Reaction with Various Initial Concentrations of L5178Y Cells.

Initial count/ml of control and inoculated cultures	Days for control cultures to reach 1.5×10^6/ml	Days for inoculated cultures to reach maximum count*	Days to >0.4 pH difference between control and inoculated cultures	Day of maximum colony yield when plated on agar†
100,000	3	3 (900,000)	3	3
50,000	4	4 (880,000)	4	4
25,000	5	4 (720,000)	4	4
12,500	5	5 (650,000)	5	5
6,250	6	5 (520,000)	5	5
3,125	7	6 (520,000)	6	6

* Numbers in parentheses are the maximum counts of inoculated cultures. Each infected tube received 10^5 L.U./ml of L.M.2 lytic mycoplasma. Each tube was sampled every day for cell count and inoculation onto "PPLO agar."

† Total yield appeared to be smaller with lower initial cell count.

cation ceased. Nevertheless, such acid cultures contained viable cells for several days even without subcultivating or feeding. Ordinarily, a 1:25 subcultivation dilution was made of all cultures when counts approached 10^6 cells per ml. This procedure was required every second or third day to maintain a high growth rate.

The specific time sequence of events following introduction of lytic mycoplasma depended upon both the dose used and the initial cell count of the culture. A typical experiment showing the effect of various initial cell counts is recorded for the L.M.2 strain in Table I and demonstrates the earlier lysis and pH change obtained when optimal cell numbers are exposed to a large amount of lytic mycoplasma.

In all cases, however, the general features of the lytic reaction were unambiguous. First, a slight degree of cellular agglutination occurred, then cessation of metabolism as evidenced by a rising pH, and finally, lysis of the cultures. At this point, when agitated, the infected tube cultures were clear with a pH of 7.5 or 7.6, while the control cultures were densely turbid with a pH of 6.9 or 7.0 (Fig. 1). If care was taken to start with optimal cell numbers and agent inoculum (multiplicity 1:1 or greater), complete clearing of the cultures was obtained. Lysis was delayed or incomplete if low multiplicities were used. Nevertheless, in many hundreds of such infected cultures emergence of resistant cells has not been observed.

Non-specific cell destruction was occasionally observed during attempts to isolate lytic agents from tissues, fresh sera, or other biological materials. Such cell destruction was distinguishable from the lytic reaction of an infectious agent since the "lytic activity" could not be serially transferred to fresh cultures of L5178Y cells.

L5178Y cell culture lysates or other materials were titered for lytic activity by

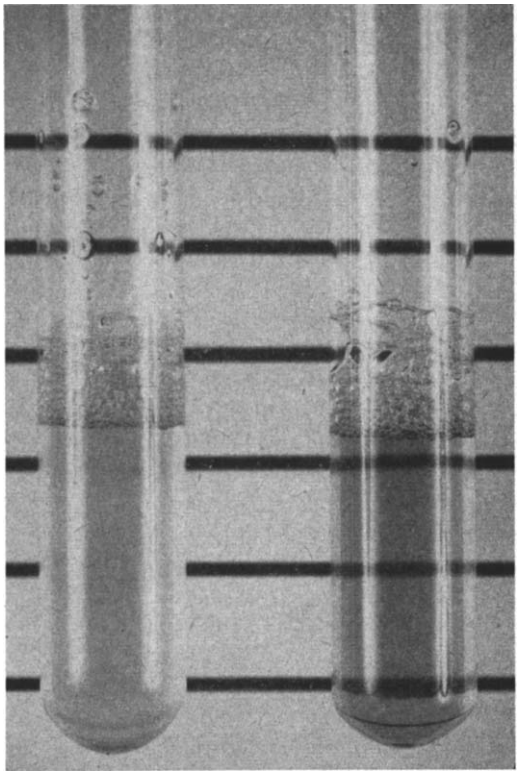

FIG. 1. L5178Y tube cultures were vigorously shaken before being photographed in order to resuspend any cells present. Culture on left is a control culture, had a cell count over 10^6 cells/ml, and was yellow (pH about 6.9). Culture on right had been inoculated with L.M.2 strain of mycoplasma 2 days previously and was red (pH about 7.5).

standard dilution-end-point technics in fresh tube cultures of L5178Y cells. Such titrations proved to be reproducible and yielded sharp end points if the cultures were subcultivated every second day for 10 days. L5178Y cell culture lysates of these agents generally titered 10^6 to $10^{7.5}$ lytic activity units (*i.e.*, $TCID_{50}$/ml) in this system.

All of the agents described have been readily carried in L5178Y cell cultures for many passages without evident change in their properties. L-1 medium without cells neither supported replication nor prevented thermal inactivation of these agents when such media controls were incubated at 37°C.

Growth of lytic mycoplasma on agar. Numerous attempts to demonstrate mycoplasma in the stock L5178Y cell line by plating samples of these cultures onto 'PPLO' agar have been negative. Spontaneous lysis of L5178Y cultures has not occurred in our experience. In addition, attempts to "induce" lytic agents in L5178Y cultures irradiated with graded doses of ultraviolet light have been negative.

However, lysates of L5178Y cultures that had been inoculated with any of these active materials (*i.e.*, passage lysates or "lytic-active" tissue cultures) when plated onto agar yielded distinct colonies up to 0.1 mm in diameter within 2 to 10 days of incubation at 37°C (Fig. 2 and 3). The colonies of the L.M.1-4 strains were typically dense, finely granular, and often lacked the classical "fried-egg" appearance. The B-7 and 12-3 strains, however, produced colonies that were similar to the appearance of such well-studied mycoplasma strains as Campo and T-5. The B-7 and 12-3 colonies also appeared more rapidly on agar than the L.M.1-4 strains.

Colonies of all strains studied retained the Dienes stain to some extent, B-7 and 12-3 being most clearly positive in this regard.

To verify the identity of the mycoplasma colonies that appeared on these agar plates with the *in vitro* "lytic activity" (that could be passed in L5178Y cells), single colony transfers were carried out with Pasteur pipettes and an inverted microscope. Such transfers of single colonies were done in series through 3 or more agar passages followed by single colony transfers to L5178Y

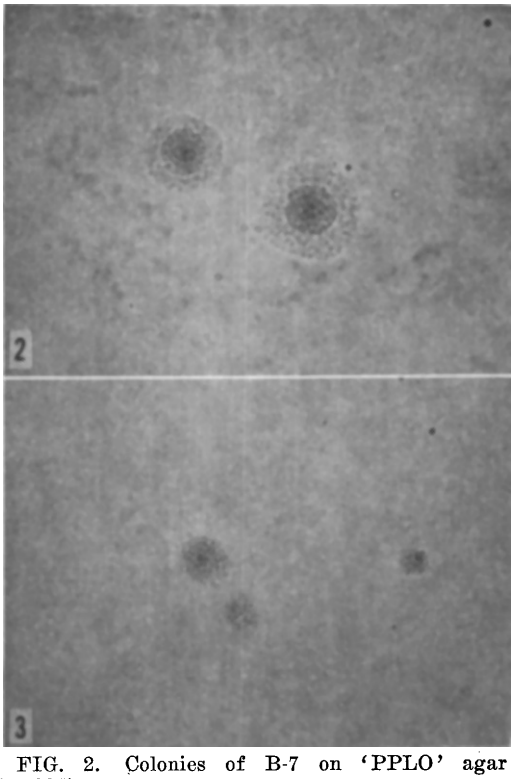

FIG. 2. Colonies of B-7 on 'PPLO' agar (× 297).

FIG. 3. Colonies of L.M.2 (a human diploid cell strain isolate) on 'PPLO' agar (× 297).

cultures. Comparable "blank agar" transfers were used as controls. All of these agar-passed mycoplasma that were derived from L5178Y cell lysates retained their lytic activity for L5178Y cells.

Lytic and non-lytic mycoplasma. Many samples of media and cells from various diploid cell strains were inoculated both into L5178Y cell cultures and onto agar plates. If colonies grew on the agar plates, several from each plate were picked under direct observation using an inverted microscope and tested for lytic activity in fresh L5178Y cultures. A significant proportion of these agar plates yielded no colonies though the corresponding L5178Y culture was positive for lytic activity. When such lysed L5178Y cultures were plated onto agar, numerous colonies developed.

Of the agar plates directly inoculated with diploid cell culture material and yielding mycoplasma colonies, many contained mixtures of lytic and non-lytic mycoplasma as tested

by single colony inoculation into L5178Y cultures. Although the 2 types of colonies were not always clearly distinguishable morphologically, their lytic activity appeared to be a stable marker: lytic mycoplasma consistently remained lytic after serial passage on agar and non-lytic mycoplasma remained non-lytic on passage.

When non-lytic mycoplasma were inoculated into L5178Y cells, the cultures showed no change in growth rate, rate of acid production, or lysis. Furthermore, these non-lytic mycoplasma could be recovered from the culture only sporadically and in low yield. Presence or absence of antibiotics did not alter the result.

The contaminated cell lines and strains contained various levels of either pure or mixed (with respect to the lytic marker) mycoplasma contamination. In general, however, those contaminated tissue cultures that yielded large numbers of colonies on direct agar plating contained primarily non-lytic mycoplasma that grew rapidly on agar. Cultures yielding only a few (or no) colonies on direct plating contained predominantly lytic mycoplasma that grew slowly on agar.

Ecology of lytic mycoplasma. Definite information concerning the occurrence of lytic mycoplasma is lacking. These agents could be isolated from only a few of the many virus pools tested (L5178Y cells are resistant to most viruses tested). Many of the additional isolates made from uninoculated tissue cultures came from the same laboratory and, therefore, might all be identical. Lytic mycoplasma have not been isolated from primary mouse, hamster or chicken embryo cell cultures, or from L cell, P388D_1, and hamster hemangioma lines carried in this laboratory. Forty-two organ extracts from 14 human fetuses were negative for lytic activity. Our results are, therefore, not inconsistent with the hypothesis that tissue culture mycoplasma originate from laboratory contamination(7).

In addition, mycoplasma strains Campo, T-5, Eaton mycoplasma, and 7 unclassified human pharyngeal isolates have been found to be non-lytic. (The Campo and T-5 strains were obtained from Dr. Harry Morton, Univ. of Pennsylvania; the human pharyngeal isolates from Dr. Robert Chanock, Nat. Inst. of Allergy & Infect. Dis.)

TABLE II. Thermostability of *B-7* Lytic Mycoplasma at Various Temperatures.

Storage temp, °C	Half-life*
−60	Approx 1 mo
10	5 days
37	7 hr
50	1.5 min

* Each determination is the average of at least 2 independent estimates.

Stability and size of lytic mycoplasma. The thermostabilities of these agents were estimated from tube dilution titrations (in L5178Y cultures) of lysates stored at various temperatures in sealed glass ampoules. Average results of at least 2 independent estimates for each temperature are tabulated in Table II for the B-7 strain. These and other results indicated that these lytic mycoplasma were not unusual with respect to their thermostability as compared to other mycoplasma (8).

Lysates of 6 strains of lytic mycoplasma were passed through Millipore filters of pore sizes 450 mμ, 300 mμ, 100 mμ and 50 mμ. Little loss in titers of filtrates that had passed 450 mμ or 300 mμ filters occurred, but there was complete loss of lytic activity in filtrates passed through the 50 mμ pore filter. Rarely could recovery be made from the filtrates of the 100 mμ filters.

Growth of lytic mycoplasma in cells other than L5178Y. The cell cultures that had yielded mycoplasma lytic for L5178Y cells were not grossly cytopathic at time of testing. Consequently, attempts were made to determine whether these agents would grow and manifest cytopathic effects in other types of cell cultures. In one series of experiments, groups of tubes of various types of cell monolayers were exposed for 2 hours to 100 $L.U._{.50}$ (lytic activity units as measured in L5178Y cell system titrations) of B-7. These cultures were then washed with media 4 times, fresh media replaced, and after 0, 3, 6 and 9 days' incubation, cultures were removed for titration in the L5178Y cell system. Titration averages for 5 different types of cell cultures are represented in Fig. 4. These cultures showed

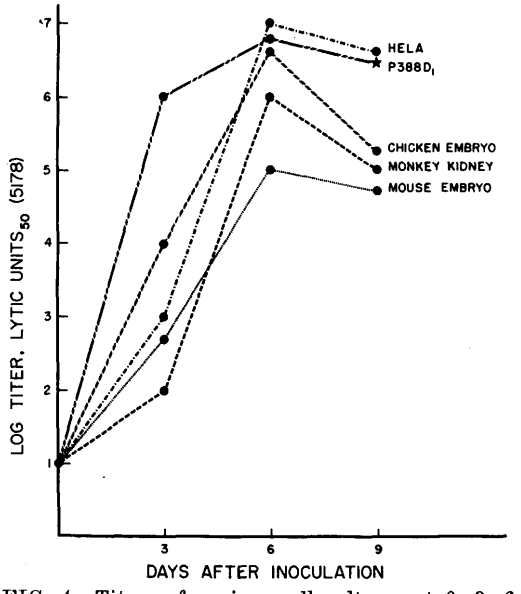

FIG. 4. Titers of various cell cultures at 0, 3, 6 and 9 days after inoculation with 100 lytic units B-7 lytic mycoplasma. Filled circles indicate cultures without evident cytopathology; star indicates cultures with advanced cytopathology.

no gross cytopathology throughout the observation period except for P388D$_1$ cultures which were completely destroyed.

These results demonstrate first, that in a variety of cell culture types, agent replication seemed to occur at comparable rates and reached similar peak titers to those attained in L5178Y cultures. Second, none of these cells showed any evident cytopathology or diminished growth rate except P388D$_1$ cells which were completely destroyed. It is noteworthy that P388D$_1$ cells originated from a DBA/2 mouse lymphoma as did L5178Y cells(9).

In addition, monolayer cultures of hamster embryo, mouse embryo (including whole mouse embryo cultures of DBA/2 mice), L cells and HeLa cells were maintained for many weeks following infection with lytic mycoplasma. Such cultures remained infected as long as they were carried. At no time was any cytopathic effect evident, and growth rates of control and infected cultures appeared equal.

In one experiment, mouse embryo cultures chronically infected for one month with B-7 were trypsinized, the cells washed 3 times with specific rabbit antiserum prepared against the B-7 agent, and dilutions of cells inoculated into L5178Y cell cultures. The results of this experiment (Table III) were interpreted as indicating that a minor proportion of these mouse cells contained the B-7 agent at any one time. Direct titrations of tissue cultures chronically contaminated with lytic mycoplasma gave variable results but generally contained 10^2 to 10^4 L.U. per ml of the whole culture.

The pattern of colonies which developed when such whole cultures were plated directly onto 'PPLO' agar suggested that most of the organisms were cell-associated: any colonies that appeared tended to be clustered around and on the residua of the dead cells.

Response of animals to lytic mycoplasma. An attempt was made to develop a serological procedure analogous to the neutralization reaction commonly used in virus studies. Rabbits were subjected to multiple injections of B-7 or 12-3 lysates or uninoculated L5178Y cells in Freund's adjuvant. The sera were collected 3 weeks after the last injection and adsorbed with L5178Y cells to reduce their toxicity for growing L5178Y cell cultures. Dilutions of such sera were incubated with 100 lytic-activity units of the various mycoplasma strains and the mixtures then added to fresh L5178Y cultures. By this method it was possible to demonstrate that a 1:60 dilution of either the anti-B-7 or the anti-12-3 serum was the highest dilution that would neutralize 100 lytic-activity units of B-7 or 12-3 respectively. The pre-immunization sera of these rabbits and the anti-L5178Y cell sera were without neutralizing ability either before or after adsorption with L5178Y cells. Furthermore, cross neutralization tests showed that either the anti-B-7 or

TABLE III. Proportion of Cells Infected in a Whole Mouse Embryo Culture, Without Evident Gross Cytopathology, Chronically Contaminated With *B-7* Lytic Mycoplasma.

No. of cells* inoculated into each of 16 L5178Y cultures	1620	162	16	1.6
Proportion of cultures positive	16/16	16/16	12/16	1/16

* Conditions of experiment described in text.

TABLE IV. Median Survival of DBA/2 × BALB/E F_1 Hybrid Mouse Groups Receiving L5178Y Cells Intraperitoneally Followed by *B-7* Lytic Mycoplasma.

	No. of L5178Y cells inoculated I.P.	Log lytic units of *B-7* inoculated	Time of *B-7* inoc	No. of animals	Median survival (days)
	15,000	none	—	5	28
	15,000	3.5	1 hr after cells	3	29
Exp 1	15,000	3.5	48 " " "	3	28
	15,000	5.5	1 " " "	3	28
	15,000	5.5	48 " " "	3	28
	1,500,000	none	—	5	18
	1,500,000	3.5	1 hr after cells	3	17
Exp 2	1,500,000	3.5	48 " " "	3	19
	1,500,000	5.5	1 " " "	3	21
	1,500,000	5.5	48 " " "	3	19

anti-12-3 serum was equally effective in neutralizing B-7 or 12-3 but that neither serum had any effect on any of the L.M.1-4 strains. It was thus concluded that B-7 and 12-3 strains were probably identical, but were different from at least some of the lytic mycoplasma isolated from tissue cultures.

Many attempts were made to demonstrate establishment of infection in rats, mice or hamsters with lytic mycoplasma, by inoculating lysates by intracerebral, intraperitoneal or subcutaneous routes into newborn or young adult animals. The animals were followed for appearance of disease, and after various periods sera were tested for neutralizing antibody and tissues for lytic activity. Recovery of the organisms from tissues or demonstration of neutralizing antibody was not achieved. No apparent disease was produced and, in particular, the inoculation of large single doses of lytic mycoplasma into DBA/2 mice was without effect, either clinical or serological.

Attempts to demonstrate in vivo *oncolysis.* The unusual cytopathogenicity of these lytic mycoplasma for both L5178Y and $P388D_1$ cells prompted investigation of the effects of these agents on growth of one of these tumor lines in genetically susceptible hosts. In one experiment, survival times of DBA/2 × BALB/C F_1 hybrid mice that had received L5178Y cells i.p. were compared with those of such mice that had received both L5178Y cells and an inoculation of B-7 mycoplasma. Results (Table IV) indicate no oncolytic effect. When several dying mice which had received B-7 were tested for neutralizing antibody against B-7, results were negative.

In a similar experiment, an additional group of mice was inoculated with L5178Y cells that had been pre-incubated for 2 hours with B-7 at a multiplicity of about 100 lytic units per cell. There was no evidence of prolongation of life in animals receiving B-7, and furthermore, the group that received cells premixed with B-7 was not protected.

During this experiment it was possible to harvest ascites fluids containing up to 10^9 cells/ml from dying animals of 3 groups: animals that had received only L5178Y cells, animals that had received L5178Y cells and B-7 separately, and those that had received pre-mixed L5178Y cells and B-7. These ascites cells were immediately suspended in L-1 media (to a count of 100,000/ml) and their sensitivity to B-7 compared to each other and to cells that had been carried in continuous culture. This was accomplished by replicate titrations of dilutions of a single aliquot of B-7 in the 4 groups of cell cultures. No differences in the sensitivity of cells having various histories could be demonstrated, and control tubes of all cell groups grew continuously thereafter.

Discussion. The mechanism of lysis observed with these lytic mycoplasma in the *in vitro* system is obviously confounded by the complete failure to demonstrate oncolysis *in vivo.* No evidence was obtained that *in vivo* protection of the L5178Y cells from lysis was attributable to production of anti-mycoplasma antibody. Growth conditions of the L5178Y cells *in vivo* were obviously different from *in vitro* conditions, since maximal cell concentrations achieved in the ascites fluids exceeded by a thousand-fold the maxi-

mum in L-1 media. It might be speculated that under *in vivo* conditions selection for resistance or autoprotective antibody formation (by the L5178Y cells) occurs. However, no evidence for any such change in susceptibility could be demonstrated in the freshly harvested ascites cells derived from cells exposed to lytic mycoplasma.

Of the many types of cell cultures found to be able to support the growth of these lytic mycoplasma only 2 types of cells were affected adversely. Both of these (L5178Y and $P388D_1$) are malignant lymphoblastic cells of DBA/2 origin. Yet massive inoculation of lytic mycoplasma into DBA/2 mice at various ages and by various routes proved innocuous and, so far as could be determined, non-infectious. In addition, whole mouse embryo cultures of DBA/2 mice were as resistant to any discernible cytopathic effects as were the other types of cell cultures tested.

The lytic reaction associated with this distinct, but as yet poorly defined, group of mycoplasma does not occur in all malignant cells, nor all murine cells. Whether it would be observed with lymphoblastic cells other than DBA/2 malignant lymphoblastic cells is not known.

Obviously, indirect mechanisms must also be considered in attempting to understand the *in vitro* lytic reaction. Destruction of the susceptible cell cultures may depend upon factors other than cellular infection, such as mycoplasma-cell competition for a critical nutrient or mycoplasma-cell interaction yielding a product toxic for the cells. These possibilities have not been explored, but it is of interest that 2 malignant murine cell lines, L-cells and $P388D_1$ cells, grown with the same media, react differently to these lytic mycoplasma.

The L5178Y cell system appears to be suitable for investigation of these distinct mycoplasma; using this system it is possible to detect, isolate and quantitate these agents with a high degree of sensitivity and reproducibility. Preliminary results also suggest that simple serological methods using the L5178Y cell culture in a neutralization test will aid the classification of these agents.

Summary. Preliminary characterization of a group of mycoplasma that are cytocidal for L5178Y and $P388D_1$ cells, but not for many other tissue culture cells, is presented. Lysis of these murine malignant lymphoblasts is a property not shared by many other mycoplasma including such agents as Campo, T-5, and Eaton mycoplasma.

Appreciation is extended to Dr. Robert Roosa, Wistar Inst., Philadelphia, Pa., who helped with the oncolysis attempts.

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Received November 12, 1962. P.S.E.B.M., 1963, v112.