Modification of the Growth of Human Mycoplasmas in Tissue Culture by Infection with Influenza Virus and Japanese Encephalitis (JE) Virus (33874)

MASAHIRO NAKAMURA AND HIROAKI SAKAMOTO
(Introduced by W. W. Ackermann)
Department of Microbiology, Kurume University School of Medicine, Kurume, Japan

Since the first report of mycoplasma contamination in tissue culture cells (1), a relationship between virus growth and the presence of mycoplasma in tissue cultures has been studied. In particular, it has been demonstrated that the growth of adenovirus, especially type 2, is greatly inhibited by mycoplasma which competes for arginine which adenovirus type 2 absolutely requires for growth in KB or other human cells (2). On the other hand, there are some reports in which the growth of mycoplasmas *in vivo* is stimulated by the virus infection (3, 4) and vice versa (5). The present paper describes the stimulation of the growth of human mycoplasmas by influenza virus and Japanese encephalitis (JE) virus infections *in vitro*.

Materials and Methods. Mycoplasmas. Three mycoplasma (M) strains, *M. pneumoniae* CL. FH. *M. hominis* type 1-C ATCC, and *M. orale* N-1 strains were used throughout the experiments. These previously cloned strains were kindly supplied by Dr. Hayflick and were propagated using the fluid medium described by him (6). The medium was supplemented with penicillin (final concentration, 1000 units/ml) and thallium acetate (final concentration, 0.2 mg/ml). The solid medium for titration was prepared similarly, except that PPLO agar (Difco) was substituted for PPLO broth (Difco).

The mycoplasmas were recovered from PPLO broth 7 or 10 days after cultivation at 37°. Mycoplasmas intended for tissue culture inoculation were sedimented from the culture medium by centrifugation at 3000 rpm for 30 min, washed with saline, and resuspended in sterile saline. The suspension usually contained 10^4 organisms/ml.

Viruses. The WS (E-22) and NWS (E-8) strains of type A influenza virus grown in the chorioallantoic cavity of embryonated eggs were used. Chorioallantoic fluids contained approximately 10^7 egg infection doses 50% (EID_{50}) of influenza virus with a hemagglutination titer (HAT) of 8192.

The Nakayama strain of Japanese encephalitis (JE) virus (M-132) propagated in the brains of 3-week-old mice was used. Virus suspensions prepared from infected brains with sterile saline contained 10^7 plaque-forming unit (pfu)/ml.

Mycoplasma assay. The number of colony-forming units (cfu)/ml of mycoplasma was determined by inoculation onto agar medium of 0.1 ml of decimal dilutions.

Virus assay. Influenza virus growth in tissue culture fluid was determined by hemagglutination titrations: 0.5 ml of twofold diluted culture fluids was added to equal volumes of 0.5% chicken erythrocytes. End points of the titrations were expressed as final dilution of virus materials which caused partial (50%) agglutination. The JE virus growth in tissue culture fluids was measured by plaque titration using chick embryo cell monolayers as described in detail elsewhere (7, 8).

Tissue culture systems for infections. In the case of mixed infections of influenza virus and mycoplasmas, the chorioallantoic membranes (CAM) of 11-day-old embryonated eggs were exclusively used as the host. Two quarter chorioallantoic membranes (60 mg) were incubated in 4 ml of Hanks culture medium containing 10% bovine serum and penicillin (100 units/ml), without streptomycin, in a 50-ml Erlenmyer flask. The inoculum to each flask was 0.2 ml of undiluted mycoplasma suspension and 0.2 ml of virus material diluted 100 times. The cultures were routinely incubated with shaking at 37°. Virus multiplication was measured by a hemag-

glutination test using 48-hr culture fluids. Mycoplasma growth was also measured by cfu on PPLO agar using culture fluids harvested every 24 hr.

In the experimental systems, *l*-arginine was added to some culture flasks at the final concentration of 1.0 m*M* according to the experimental design. During incubation, culture fluids of all of the bottles and flasks were adjusted periodically to approximately pH 7.4–7.6 with sterile NaOH, to eliminate the acidity which would inactivate virus and mycoplasmas. For mixed infections with JE virus and mycoplasmas, PS (porcine kidney stable) cell monolayers previously found to have no mycoplasma contamination were used as the host. The cell monolayers in each bottle were washed twice with phosphate balanced salt solution and maintained in 10 ml of Hanks culture medium containing 2% rabbit serum with no neutralization antibody against JE virus, and penicillin, but no streptomycin. Two-tenths ml of undiluted mycoplasma suspensions and JE virus diluted 10^{-4} were inoculated both at different intervals and simultaneously to the cell monolayers, and incubated at 37°. Virus and mycoplasma growth in the culture fluids were measured every 24 hr after inoculation by the method mentioned above.

Results. Effects of mycoplasmas on the growth of influenza virus in vitro. A relationship between mycoplasma growth and the multiplication of the WS and NWS strains was studied. Each virus was inoculated to chorioallantoic membranes (CAM) in tissue culture 24 hr before, simultaneously, and after 24 and 48 hr of mycoplasma inoculation. The normal growth curves of the mycoplasma were established without viral infection. The multiplication of virus was determined in the membrane with and without mycoplasma infection. The results obtained are shown in Fig. 1. In the absence of virus, *M. pneumoniae* and *M. orale* could not be recovered from the tissue culture fluid 24 and 48 hr after inoculation, respectively, but *M. hominis* gradually increased. The growth of influenza virus strains tested, especially the WS strain, was greatly inhibited by mixed infection with *M. pneumoniae* when the viruses were inoculated 2 days after mycoplasma inoculation (Fig. 1, left).In the case of the NWS strain (Fig. 1, right), a similar tendency was observed and a slight inhibitory effect of *M. orale* on the virus multiplication also was noted. However, *M. hominis* had no such effect and the growth of the viruses was not inhibited when the viruses were inoculated before or at the same time as the mycoplasma.

The effects of influenza virus infections on the growth of mycoplasma in vitro. Next the growth of mycoplasmas and viruses were both measured in a mixed infection. The WS strain was inoculated into the flask containing CAM in Hanks medium 2 days after inoculation of *M. pneumoniae* or *M. orale*. After 2-days' incubation at 37°, the virus, as well as mycoplasma in the fluid, was titrated. The results are shown in Table I. The virus growth was inhibited by preinfection of *M. pneumoniae*, but not by *M. orale*, whereas the growths of *M. pneumoniae* and *M. orale* were stimulated by post infection of the WS strain. When l-arginine was added to the Hanks medium at a concentration of 1.0 m*M*, the growth of the virus was not inhibited by mycoplasma. However, growth of mycoplasmas was still stimulated as in the Hanks medium. A similar finding as that obtained in the WS-*M. pneumoniae* infection system was observed in the case of the NWS-*M. pneumoniae* infection. In Hanks medium containing arginine, (Table II), the growth of *M. pneumoniae* was stimulated by the NWS strain, but not in the Hanks medium without arginine. Inhibition of the NWS strain by *M. pneumoniae* did not occur in the presence of arginine.

The effect of JE virus infection on the growth of mycoplasma. First of all, the growth curves of *M. orale* and JE virus in PS cells were separately determined. The results are demonstrated in Fig. 2. The growth of *M. orale* started 4 days after the negative phase and lasted for 3 days after inoculation in either Hanks or Hanks medium containing arginine. The JE virus started to multiply 2 days after incubation in the Hanks and 1 day after incubation in the Hanks medium con-

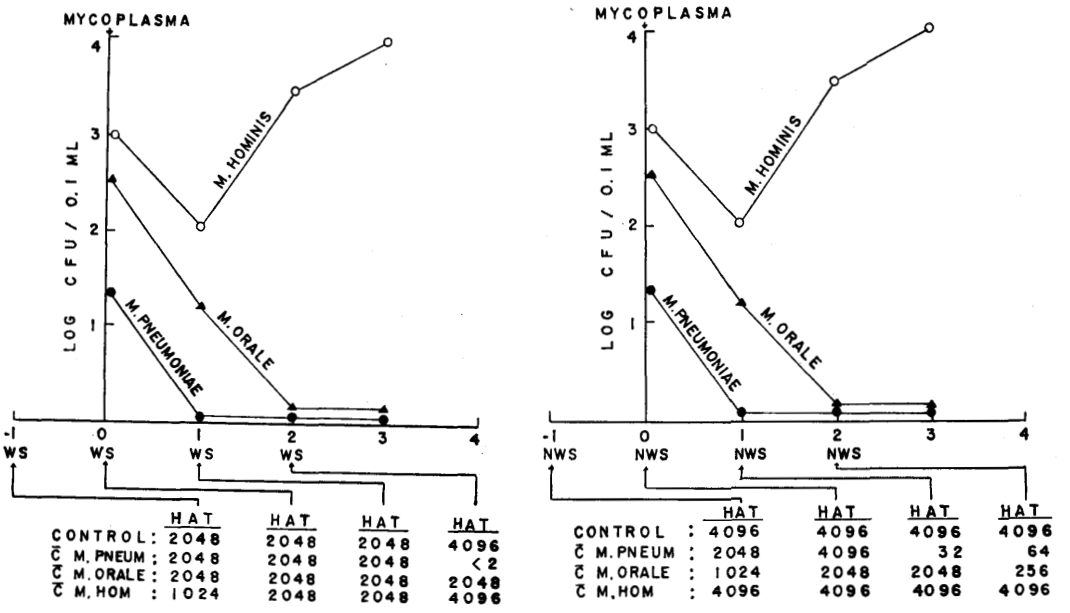


FIG. 1. Effect of mycoplasma on the growth of influenza virus strain WS (left) and NWS (right) in cultures of chorioallantoic membrane. The abscissa indicates days after addition of mycoplasma to cultures. The curves describe the growth of mycoplasma in cultures of chorioallantoic membranes that were not infected with influenza virus. The arrows indicate the day of viral inoculation into replicate cultures while the position of the data indicates the day the viral titer (HAT) was determined.

taining arginine, respectively. The cytopathogenic effect was observed 4 days after inoculation.

The results obtained from the experiment in which PS cells were simultaneously infected with JE virus and *M. orale* are shown in Fig. 3A. *M. orale* growth was inhibited by mixed infection with JE virus, but the negative phase which was observed in normal growth disappeared; *M. orale* grew 2 days after inoculation. On the other hand, there was no significant difference between the growth pattern of JE virus in the experimental group and in the normal group.

TABLE I. Growth of Mycoplasmas and Influenza Virus (WS) in CAM *in Vitro*.

Infection of 11-day-old CAM			Growth	
First inoculation	Second inoculation[a]	Tissue culture medium[b]	WS (HAT)	Mycoplasma (cfu/0.1 ml)
—	WS	H	2048	—
M. pneum.	—	H	—	3.0
"	WS	H	4	3.3
M. orale	—	H	—	3.8
"	WS	H	2048	4.9
—	WS	H + A	2048	—
M. pneum.	—	H + A	—	3.6
"	WS	H + A	2048	3.9
M. orale	—	H + A	—	3.3
"	WS	H + A	1024	5.0

[a] Interval between first and second inoculation was 2 days.

[b] H = Hanks; H + A = Hanks containing 1.0 m*M* arginine.

Next, the results obtained by inoculation of PS cells with JE virus and *M. orale* at different times are indicated in Fig. 3B–D. When the JE virus was introduced 2 days *after* inoculation of *M. orale* both the virus and mycoplasma grew to the same degree as normal. However, when the virus was inoculated into the cells 1 or 2 days *before* mycoplasma inoculation, curious growth patterns of mycoplasma were noted. There was no negative phase of growth. The mycoplasma grew rapidly 24 hr after inoculation. However, the growth patterns of JE virus were not affected by mycoplasma infection.

Discussion. The relationship between virus and mycoplasma in natural infections is still obscure. However, it seems that mycoplasma has certain significant roles in viral respiratory diseases. In previous works (9), it was demonstrated that the death rates of mice infected with influenza virus were enhanced by mycoplasma infection. The present paper describes results obtained in mixed infections of influenza virus with mycoplasma in a tissue culture system. The results obtained revealed that influenza virus growth was inhibited by mycoplasma infection, while the mycoplasma growth was stimulated by virus infection. The former might be due to depletion of arginine in the tissue culture medium by mycoplasma metabolism; as the inhibition of the virus was removed by addition of arginine. It is noted, therefore, that arginine is a necessary nutrient for influenza virus multiplication. This finding also was supported by investigations with other viruses such as adenovirus (2), infectious bovine rhinotracheitis virus (10), Rous sarcoma virus (11) and others (12, 13) with mycoplasma infections in tissue culture systems.

On the other hand, it was obvious that the growth of *M. pneumoniae* as well as *M. orale* was enhanced by infections with influenza viruses in the medium containing arginine.

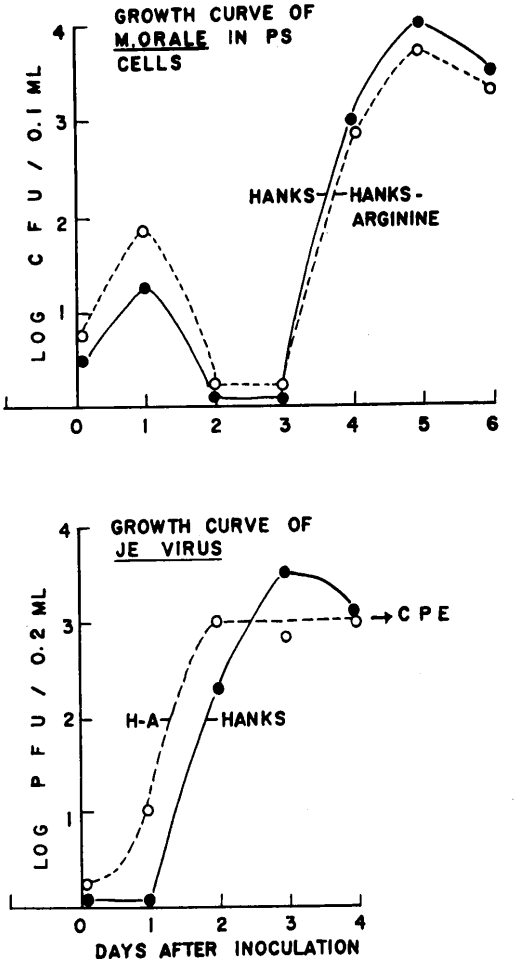
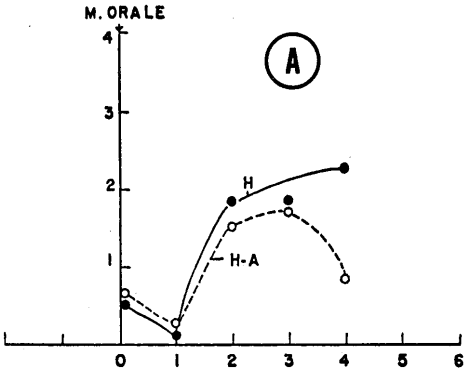
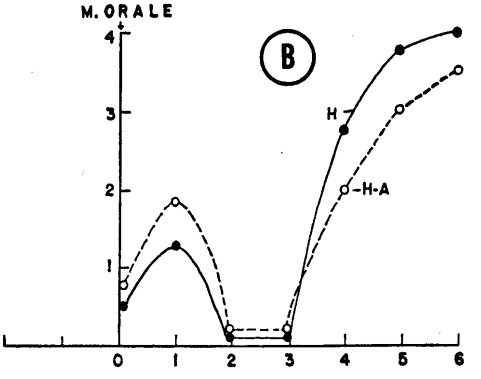


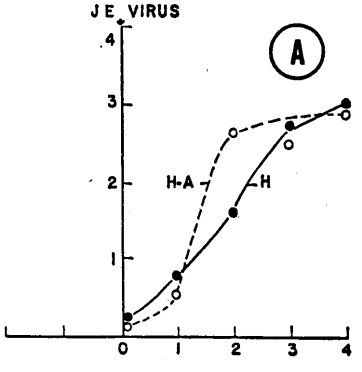
FIG. 2. Normal growth curves of *M. orale* and JE virus in the PS cells in Hanks, and Hanks medium containing *l*-arginine 1.0 m*M*.

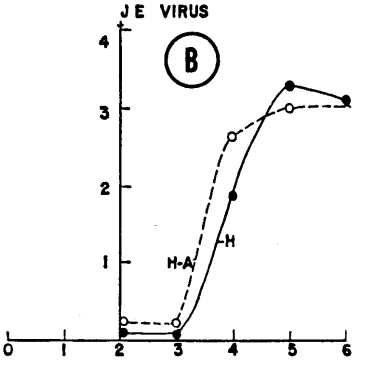
These findings might suggest that the growth of mycoplasmas was enhanced by using metabolic products of arginine utilized during influenza virus multiplication, because the growth of mycoplasma itself was not stimulated by arginine, and arginine is a necessary factor for influenza virus multiplication. However, the mechanism of growth stimula-

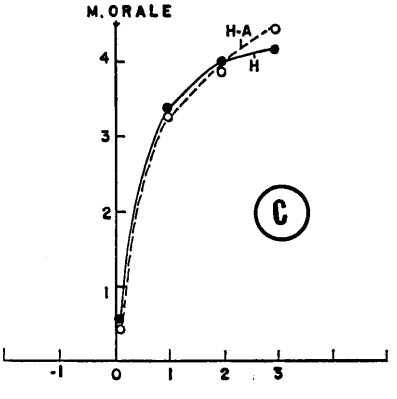
FIG. 3. Effect of JE virus on the growth of *M. orale* in PS cell cultures. Cultures were inoculated in Hanks medium (H) or in Hanks containing *l*-arginine, 1.0 m*M* (H-A). The ordinates represent growth of *M. orale* in log cfu/0.1 ml and of JE virus in log pfu/0.2 ml. The abscissa in all graphs represents days after addition of *M. orale* to the cultures. (A) *M. orale* and JE virus added simultaneously. (B) JE virus added 2 days after *M. orale*. (C and D) JE virus added 1 and 2 days before *M. orale*, respectively.

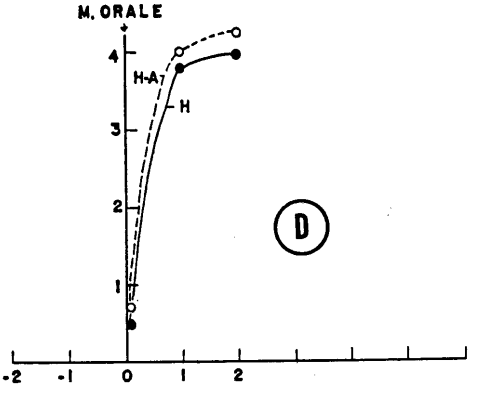

M. ORALE
A
H
H-A

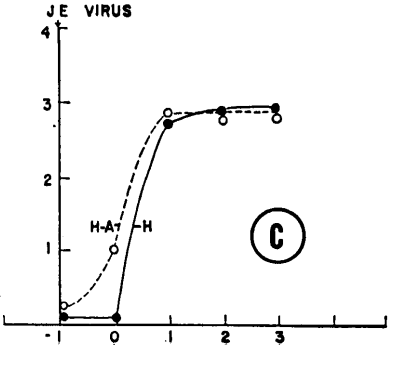

M. ORALE
B
H
H-A


J E VIRUS
A
H-A
H


J E VIRUS
B
H-A
H


M. ORALE
H-A
H
C


M. ORALE
H-A
H
D


J E VIRUS
H-A
H
C

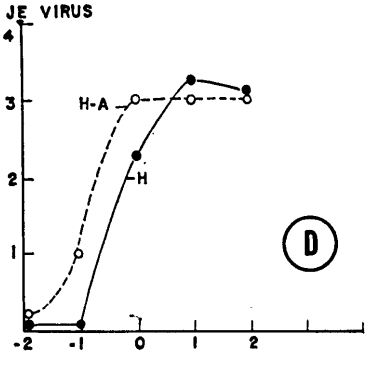

J E VIRUS
H-A
H
D

TABLE II. Growth of *M. pneumoniae* and Influenza Virus (NWS) in CAM *in Vitro*.

Infection of 11-day-old CAM			Growth	
First inoculation	Second inoculation[a]	Tissue culture medium[b]	NWS (HAT)	Mycoplasma (cfu/0.1 ml)
—	NWS	H	1024	—
M. pneum.	—	H	—	4.7
"	NWS	H	256	4.4
—	NWS	H + A	512	—
M. pneum.	—	H + A	—	3.0
"	NWS	H + A	1024	5.0

[a] Interval between first and second inoculation was 2 days.
[b] H = Hanks; H + A = Hanks containing 1.0 m*M* arginine.

tion for mycoplasma by influenza virus is not clear.

In the case of the JE virus and mycoplasma system, the virus growth was not influenced by mycoplasma infection. However, the mycoplasma growth patterns were significantly changed by preinfection of the PS cells by JE virus. This might be due to the utilization of cell components or other components associated with the course of JE virus multiplication, although visible cell destruction by the virus was not observed. This was because there was not stimulation of mycoplasma growth when the virus was inoculated *after* mycoplasma infection.

From the results described in previous and present papers, it is of interest to note here that there were different growth patterns of mycoplasma in mixed infection with influenza virus and with JE virus, *i.e.*, *M. pneumoniae* and *M. orale* growths were enhanced by postinfection with influenza virus, while *M. orale* growth was not enhanced by postinfection with JE virus. It might be thought that these different findings were caused by differences in the growth characteristics of myxovirus and arbovirus, even though both are RNA viruses, and by differences in the properties of the cells used for tissue culture.

Summary. The growth of type A influenza virus in tissue cultures of chorioallantoic membrane was inhibited by *M. pneumoniae* when the latter was inoculated 2 days before the virus. However, this inhibition was reversed by addition of *l*-arginine. On the other hand, the growth of mycoplasma was stimulated by influenza virus infection, especially in the culture medium containing arginine. In the case of the JE virus growing in PS cells, the virus was not affected by *M. orale* infection. On the contrary, the growth patterns of *M. orale* were modified by JE virus infection, particularly when the virus was inoculated 1 or 2 days *before* mycoplasma infection. However, no modification of growth pattern of *M. orale* was observed when the virus was inoculated after mycoplasma infection.

1. Robinson, L. B., Wichelhausen, R. H., and Roizman, B., Science **124**, 1147 (1956).
2. Rouse, H. C., Bonifas, V. H., and Schlesinger, R. W., Virology **20**, 357 (1963).
3. Nelson, J. B., J. Exptl. Med. **106**, 179 (1957).
4. Schauwecker, R., Schweiz. Z. Allgem. Pathol. Bakteriol. **10**, 714 (1947).
5. Findlay, G. M., Klieneberger, E., Maccallum, F. O., and Mackenzie, R. D., Lancet ii, 1511 (1938).
6. Hayflick, L., Texas Rept. Biol. Med. **23**, 285 (1965).
7. Nakamura, M. and Ueno, Y., Proc. Soc. Exptl. Biol. Med. **113**, 1037 (1963).
8. Porterfield, J. S., Nature **183**, 1069 (1959).
9. Nakamura, M. and Sakamoto, H., 1968, unpublished data.
10. Afshar, A., J. Gen. Microbiol. **47**, 103 (1967).
11. Somerson, N. L. and Cook, M. K., J. Bacteriol. **90**, 534 (1965).
12. Armstrong, D. Henle, G., Somerson, N. L., and Hayflick, L. J. Bacteriol. **90**, 418 (1965).
13. Butler, M. Q. and Leach, R. H., J. Gen. Microbiol. **34**, 285 (1964).

Received Nov. 21, 1968. P.S.E.B.M., 1969, Vol. 131.