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REPORT NUMBER 3

THE RELATIONSHIP BETWEEN MYCOPLASMA SPECIES AND SELECTED RESPIRATORY VIRUSES (ADENOVIRUS, INFLUENZA VIRUS AND RHINOVIRUS)

FINAL REPORT

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SUMMARY

Mycoplasma pneumoniae, as well as certain PPLO growth medium components (PPLO broth and fresh yeast extract) stimulate rhinovirus (type 1A, strain 2060) ribonucleic acid (RNA) synthesis. The effect of mycoplasma on the formation of new virions has not been resolved, but it appears there is no increase of rhinovirus titers in the presence of M. pneumoniae compared to virus grown in the absence of mycoplasma.

Further studies of Rhinovirus-RNA synthesis suggested that with greater input multiplicities of infection (IM) of virus there was more rapid incorporation of the phosphorylated nucleotides into RNA, while at the lower IM's, there was phosphorylation of the nucleotides in the absence of comparable incorporation. In these investigations, the kinetics of synthesis of rhinovirus RNA was examined in actinomycin D-treated KB cells by assaying for both trichloracetic acid-precipitable and TCA-soluble incorporation after the addition of ^{3}H-uridine to infected cell systems. Viral RNA was first detected between three and four hours, and reached peak levels at eight to nine hours post-infection. Infectivity studies demonstrated that cell-associated virions in infected cells became detectable between four and five hours and reached peak titers at ten hours post-infection. Viral RNA synthesis, therefore, precedes the appearance of the first virions by less than an hour and appears to terminate approximately one hour before the maximal viral yield is attained. Selected IM's were studied and it was found that the rate of incorporation of H^{3}-uridine into TCA-precipitable material (RNA) increased with increasing IM of rhinovirus. Incorporation into TCA-soluble material (phosphorylated nucleotides) decreased with increasing IM's.

Rhinovirus-RNA synthesis was increased in the presence of M. pneumoniae, however, no such stimulatory effect was observed with another picornavirus, polio virus type 1. In fact, the polio virus-RNA synthesis (as measured by ^{3}H-uridine uptake in the presence of actinomycin D) appeared to be inhibited in HeLa and KB cells infected with mycoplasma.

Another difference between polio virus and rhinovirus was observed. Previous studies by Fletcher and Platt (Arch. Ges. Virus forschung 25:1, 1968) indicated the ability of human gingival cells to replicate rhinovirus. In contrast, polio virus is capable of attaching to human cells of gingival origin, but it is unable to complete its replication cycle due to the absence of viral RNA synthesis.

Adenovirus type 4 and M. pneumoniae interactions measured by tritiated-thymidine uptake showed greater DNA synthesis as compared to cells infected with virus alone. The positive identity of the labeled DNA (cellular, mycoplasma or virus) was investigated using Sepharose 2B separation. This separation of ^{3}H-thymidine labeled adenovirus type 4 was accomplished at approximately 40 ml of elution volume. M. pneumoniae particles because of their large size were eluted in the void volume. The results of these extensive studies showed no quantitative difference in labeled adenovirus recovered, whether the virus was grown in the presence of or absence of mycoplasma.

Finally, the interaction of M. pneumoniae and influenza A/PR8 virus was observed in 1,308 mice. These combinations of mycoplasma and influenza appeared to produce earlier symptoms and deaths than observed in mice infected with virus alone.

Infection of KB cells with herpes simplex virus prior to rhinovirus infection resulted in inhibition of rhinovirus replication. This was

demonstrated by reduction in both rhinovirus yield and ^{3}H-uridine uptake. The two viruses when inoculated simultaneously produced strong interference resulting in reduction of yield of both viruses. In contrast to the effect produced by rhinovirus, M. pneumoniae stimulated herpes simplex virus yields as compared to mycoplasma-free systems.

Finally, Actinomycin D (0.019 μg/ml) inhibited M. pneumoniae ^{3}H-uridine uptake by 73 percent, and inhibited ^{3}H-thymidine uptake by 50% at a concentration of 0.625 μg/ml. This clearly indicated the ideal nature of actinomycin D in mycoplasma-rhinovirus-cell systems for inhibiting all but viral-RNA synthesis.

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INTRODUCTION

In the past three years numerous reports have been published on the effect of mycoplasma on cell systems, and mention has been made of the interaction of these agents with some viruses (Stanbridge, Bact. Rev. 35: 206, 1971). One of these studies established a method for measuring the effect of mycoplasma on picornavirus-RNA synthesis (Fletcher, Milligan, and Albertson, Folia Microbiol. 15:325, 1970; and Milligan and Fletcher, Antimicrob. Ag. Chemother., 1969, p. 196).

Despite this interest much basic information is missing and any scientific discipline must progress to a reasonable state of knowledge before it can be put in order. In this regard this report describes the interaction of selected respiratory viruses and Eaton's agent. For example, enhancement of rhinovirus-RNA synthesis in *Mycoplasma pneumoniae* treated KB cells was demonstrated, and in contrast the inhibition of poliovirus-RNA synthesis in similar treated cell systems. In addition, basic studies concerning the action of rhinovirus on cell systems are explained in terms of nucleotide phosphorylation and RNA incorporation of ^{3}H-uridine.

Because of the importance of other selected respiratory viruses (adenovirus and influenza virus) on the military population these agents were investigated. In particular, the effect of mycoplasma on adenovirus ^{3}H-thymidine uptake and influenza virus infections of mice. For comparison with adenovirus-mycoplasma systems another DNA virus, herpes simplex virus, was employed.

MATERIAL AND METHODS

Viruses: Rhinovirus type 1A strain 2060, adenovirus type 4, polio virus type 1. and herpes simplex virus type 2 were procured from the American Type Culture Collection. Influenza A/PR8 virus and influenza virus B/Lee were obtained from Lederle Laboratories, Pearl River, N.Y. Detailed descriptions of these viruses were reported in Annual Reports 1 (1969) and 2 (1970), "The Relationship Between Mycoplasma Species and Selected Respiratory Viruses (Adenovirus, Influenza Virus and Rhinovirus).

Mycoplasma: *M. pneumoniae* (Eaton's agent) was also obtained from the ATCC and described in the above mentioned reports.

Viral DNA and Viral RNA Synthesis: Procedure for measuring viral incorporation of ^{3}H-thymidine and ^{3}H-uridine was described in Annual Reports 1 and 2.

Influenza Virus (In Vivo and In Vitro)

a. Animal Studies: Influenza A/PR8-*M. pneumoniae* studies were conducted using 3 week old Swiss-Webster mice (male and female, approximately 15 gms each). The mice were first inoculated while under slight ether anesthesia, by the intranasal instillation of 0.05 ml volumes of *M. pneumoniae* or BME. These inoculations were made in separate groups of mice at 4, 3, 2, and 1 days before the virus inoculation and in some cases in combination with the virus. Each mouse was infected while under slight ether anesthesia, by the intranasal instillation of 0.05 ml volumes of appropriate dilutions of influenza virus stock.

b. Gingival Cell Monolayers: Gingival cells, passed approximately 520 times since they were obtained from Smulow and Glickman (PSEBM 121: 1294, 1966), were utilized to determine their ability to replicate influenza virus. Serial passage of influenza virus was made in these

systems and viral growth determined by hemagglutination titration (Salk, J. Immunol. 49:87, 1944) and by hemadsorption (Shelokov, PSEBM 97:802, 1958).

Sepharose Separation of Adenovirus: Briefly, the method used consisted of adenovirus infected KB cell monolayers previously infected (minus 48 and minus 24 hours) with M. pneumoniae. At the time of virus additions, ^{3}H-thymidine (1μc/ml) was added, all cultures were incubated at 37°C until a 4+ viral-produced CPE was observed, and then all systems were subjected to 6 repetitions of freezing and thawing. Cell debris was removed by low speed centrifugation (4000 RPM, 15 mins), the supernatant fluid was collected and centrifuged at high speed (50,000 RPM, 120 mins). The pellet was drained and suspended in 1 ml of Earle's BME (2% calf serum), and this suspension (sample volume, 1 ml) was placed on a sepharose bed (2.1 x 56 cm) using an eluant of 0.002 M sodium phosphate buffer, pH 7.2, and 0.15 M sodium chloride (Flow rate: 2 ml/cm^2 hr). This procedure was utilized by Bengtsson and Philipson (Biochim. Biophys. Acta 79:399, 1964) to purify animal viruses. The adenovirus separation on sepharose was confirmed by infectivity determinations in KB cell systems.

Herpes Simplex Virus-Respiratory Agents: KB cell monolayers were infected with 0.1 ml herpes simplex virus ($TCID_{50}=10^7$/ml) 48 and 24 hours prior to rhinovirus inoculation. Rhinovirus inoculations (1 ml, $TCID_{50}=10^7$/ml) were made in the presence of actinomycin D (10μg/ml), and pulse-labeled for 1 hour with 2μc/ml of ^{3}H-uridine at selected times during rhinovirus replication. Radioactive samples were processed as described by Fletcher, Milligan and Albertson (Folia Microbiol. 15:325, 1970). All counts/min were corrected by subtracting appropriate incorporation of tissue or tissue-herpes virus controls. The converse experiment (pre-

infection with rhinovirus) was not conducted because herpes rhinovirus-cell systems cannot be selectively inhibited to discriminate the herpes virus uptake of tritiated-thymidine from that of the cell system.

Similar studies, based on viral-produced cytopathic effect were undertaken. Each sample was divided into three sub-samples, one treated with ethyl ether for 5 minutes in an ice bath (inactivates herpes simplex virus), the second treated with rhinovirus specific antiserum for 30 minutes at room temperature (neutralization of rhinovirus), and the third not treated. Initially, KB cell monolayers were infected with herpes simplex virus 48 and 24 hours prior to rhinovirus inoculation (incubated at 35°C) and at the time of rhinovirus inoculation (zero time). Rhinovirus was inoculated in all cell cultures (cell cultures pre-infected with herpes virus and uninfected cell cultures) at an input multiplicity of 40. After the infected cultures were incubated for 24 hours at 33°C, they were subjected to 2 repetitions of freezing and thawing, each sample was then separated into 3 sub-samples, treated as described above, and titered for herpes simplex virus and rhinovirus in KB cells.

Mycoplasma-Actinomycin D Treatment: Because of extreme sensitivity of *M. pneumoniae* to actinomycin D, the effect of different concentrations of this agent on mycoplasma was examined. The actinomycin D was added to the *M. pneumoniae* monolayers, the treated mycoplasma systems incubated for one hour, followed by a one hour pulse of ^{3}H-uridine (2.5μc/ml) or ^{3}H-thymidine (4μc/ml) at 37°C. Viability of the mycoplasma was also determined after exposure to the chemical agent by demonstrating growth on PPLO-medium.

RESULTS

In order to glean as much information as possible from the labeling experiments an assay of input counts, unincorporated label and saline rinses were examined. Pulse labeling experiments were performed and the aforementioned parameters were examined (Table 1). Seventy-eight to 86 percent of the added label was accounted for. The only meaningful data obtained was that of acid-precipitable and acid soluble incorporation. Unincorporated label and label in the saline rinses were of such great magnitude that differences were not significant among the experimental groups.

Further studies, described in part in Annual Report 2, 1970, and at the 1971 ASM Meeting (Bact. Proc., p. 172, 1971), of rhinovirus-RNA synthesis suggested that with greater input multiplicities of infection (IM) of virus there was more rapid incorporation of the phosphorylated nucleotides into RNA, while at the lower IM's, there was phosphorylation of the nucleotides in the absence of comparable incorporation. In these investigations, the kinetics of synthesis of rhinovirus 2060 RNA was examined in actinomycin D-treated KB cells by assaying for both trichloracetic acid (TCA)-precipitable and TCA-soluble incorporation after the addition of ^{3}H-uridine to infected cell systems. Viral RNA was first detected between three and four hours, and reached peak levels at eight to nine hours post-infection. Recent infectivity studies demonstrated that cell-associated virions in infected cells became detectable between four and five hours and reached peak titers at ten hours post-infection. Viral-RNA synthesis, therefore, precedes the appearance of the first virions by less than an hour and appears to terminate approximately one hour before

Table 1. Counts per minute associated with: unincorporated label; saline rinses; acid-precipitable and acid-soluble material in pulse-labeling experiments with uridine-5-H^3 in *Mycoplasma pneumoniae* pre-inoculated cells infected with rhinovirus.

		TOTAL CPM ACCOUNTED FOR[a]				
MP	Rhinovirus	Unincorporated label	Saline rinses	Acid-precipitable	Acid-soluble	% Label[c] Recovered
+	+	26,315,550 (96)[b]	902,320 (3.3)	22,483 (0.08)	34,483 (.12)	86
-	+	26,321,000 (96)	843,750 (3.1)	16,917 (0.06)	29,948 (.11)	86
-	-	24,029,140 (97)	788,150 (3.2)	14,785 (0.06)	61,856 (.25)	78

(a) The total incorporation of label into various fractions over an 11 hour experimental period.

(b) The numbers in parentheses represent the %-age of total label recovered.

(c) %-age of added label recovered.

the maximal viral yield was attained. Selected IM's were studied and it was found that the rate of incorporation of H^3-uridine into TCA-precipitable material (RNA) increased with increasing IM of rhinovirus. In contrast, incorporation into TCA-soluble material (phosphorylated nucleotides) decreased with increasing IM's.

The number of cells infected at input multiplicities of 40, 4, and 0.4 $TCID_{50}$/cell was determined. Following a 60 minute adsorption period, unadsorbed virus was removed, maintenance medium was added to the infected monolayers, and the infected monolayers were then replaced on the roller drum at 33°C and viral replication was allowed to proceed for 12 hours (i.e. the time required for one replication cycle). Monolayers were then trypsinized and trypan blue was added. Following an additional 30 minute incubation period of the trypan blue-stained cells at 37°C, cell counts were made. The number of stained (infected) and unstained (non-infected) cells were enumerated in a haemocytometer. The results of these experiments are illustrated in Table 2. At an input multiplicity of 40 $TCID_{50}$/cell, 96% of the cells were infected, at a ten-fold lower input multiplicity 68% of the cells were infected. At an input multiplicity of 0.4 $TCID_{50}$/cell 46% of the cells were infected and at an input multiplicity of 0.04 $TCID_{50}$/cell, 18% of the cells were infected.

On the basis of virus growth curves, virus RNA synthesis curves, attachment studies, and studies determining the number of cells infected at varying input multiplicities of infection, the most efficient growth cycle occurred if cell monolayers were infected at an input multiplicity of 40 $TCID_{50}$/cell.

The effect of *M. pneumoniae* on KB cells was investigated. KB cell monolayers were divided into two groups. One group of KB cell monolayers

Table 2. Number of cells infected at varying input multiplicities of Rhinovirus 2060.

Input multiplicity RV 2060	# Cells not Infected	# Cells Infected	# Cells Infected
40	3.0×10^4	4.9×10^5	96
4	1.7×10^5	3.5×10^5	68
.4	2.8×10^5	2.4×10^5	46
.04	4.3×10^5	9.4×10^4	18

was inoculated with mycoplasma at an input multiplicity of 8 acid-forming units (AFU)/cell, while the control group was sham-inoculated. Mycoplasma-inoculated and control cell systems were incubated at 33°C, and at intervals, three tubes from each group were removed, trypsinized, and cell counts were performed using trypan blue as an indicator of cell viability. The results (Table 3) show that there was little difference in the number of viable cells until 124 hours post-mycoplasma inoculation. From 124 hours until 172 hours post inoculation of mycoplasma there was a gradual decrease in the number of viable cells as compared to the sham-inoculated controls. This decrease in viability was visibly indicated by a detachment of the cells from the mycoplasma inoculated KB cell monolayers beginning at the butt of the tube and extending approximately 1/3 of the way up the cell monolayer.

M. pneumoniae was demonstrated to enhance rhinovirus-RNA synthesis (Fig. 1). However, PPLO growth medium was also capable of stimulating viral RNA synthesis in the presence of *M. pneumoniae*. The stimulation produced by *M. pneumoniae* in PPLO growth medium and by *M. pneumoniae* alone was 1.37 and 1.20 fold respectively. In contrast, PPLO growth medium alone seemed to exert no effect. The stimulatory effect of the components of the PPLO growth medium was further investigated by Fletcher *et al.* (1970) and could be attributed to the fresh yeast extract and Difco PPLO broth. Since it was shown that the components of the PPLO growth medium could stimulate viral RNA synthesis, in all future experiments *M. pneumoniae* (free of PPLO growth medium) was employed.

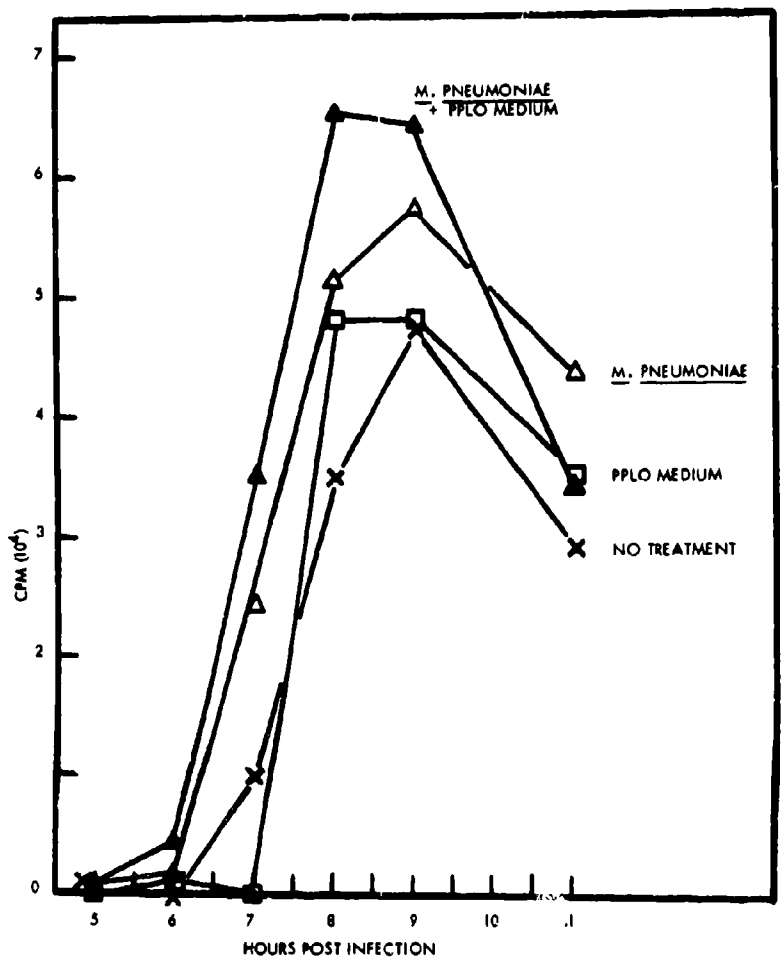
After the initial observation of 27% rhinovirus-RNA stimulation in *M. pneumoniae* treated cells, there was a question as to whether this difference was statistically significant. After performing the same

TABLE 3. Effect of Mycoplasma pneumoniae on KB cell viability. Average of three tubes per group per time interval tested.

	Cell counts at following times post-MP-G[a]							
MP	12 hours	45 hours	68 hours	76 hours	100 hours	124 hours	148 hours	172 hours
+	1.4×10^6	1.7×10^6	1.8×10^6	1.9×10^6	1.6×10^6	1.4×10^6	1.4×10^6	0.8×10^6
-	1.4×10^6	1.5×10^6	2.0×10^6	2.0×10^6	1.7×10^6	1.8×10^6	1.7×10^6	1.3×10^6

(a) Cell count prior to inoculation of tubes of Group A with Mycoplasma pneumoniae = 1.5×10^6

Fig. 1. Enhancement of rhinovirus-RNA synthesis (^{3}H-uridine uptake) by *Mycoplasma pneumoniae* and *M. pneumoniae* plus PPLO medium.



experiment several times, the "t" test of significance between two sample means ($\bar{X}_1$ and $\bar{X}_2$) with paired variates and 3 degrees of freedom was applied (Batson, An Introduction to Statistics in the Medical Sciences, Burgess Publishing Co., Minnesota, 1958). The peak of tritiated-uridine incorporation in M. pneumoniae pre-inoculated and un-inoculated cells were compared and the significance was <0.05>0.02. The stimulation of rhinovirus-RNA synthesis when cells were pre-treated 12 hours prior to viral infection with M. pneumoniae was significant.

The possibility existed that the maintenance medium in which the M. pneumoniae were suspended contained a substance stimulatory for rhinovirus-RNA synthesis. To determine whether or not the mycoplasma were necessary, a portion of the M. pneumoniae stock was passed through a millipore filter (pore size=0.1 μ). A group of cell monolayers were treated with this mycoplasma free-filtrate 12 hours prior to rhinovirus infection, and control and filtrate-treated monolayers were assayed for tritiated-uridine incorporation after rhinovirus infection. The results showed that the filtrate alone was not capable of stimulating rhinovirus-RNA synthesis, but that viable mycoplasma were necessary for stimulation.

The effect of different times of inoculation and different input multiplicities of M. pneumoniae on the virus yield was investigated (Table 4). The virus yield includes both extracellular and cell-associated virus. The data indicated that when mycoplasma was pre-inoculated into KB cell systems 12 hours prior to virus infection (input multiplicity of 40 $TCID_{50}$/cell) there was no significant change in the final virus yield. At this input multiplicity of virus, the final virus yield represents a single cycle of virus growth. When mycoplasma was pre-inoculated 12 hours prior to virus infection (input multiplicity of 4 $TCID_{50}$/cell) there was no

Table 4. Effect of *Mycoplasma pneumoniae* pre-inoculation on virus yield from rhinovirus 2060 infected KB cell monolayers.

Time of MP	IM MP	IM RV	Length of Incubation	Virus Yield $Log_{10}TCID_{50}/ml$	Difference From Control (Log_{10})
-12 hr	8	40	12.5 hr	6.6	0
-12 hr	50	40	12.5 hr	6.8	0.2 (+)
------	-	40	12.5 hr	6.6	---
-12 hr	8	4	57.5 hr	7.3	0.18 (-)
-12 hr	50	4	57.5 hr	6.8	0.67 (-)
------	-	4	57.5 hr	7.5	----
-12 hr	8	.4	80.5 hr	7.5	0.19 (-)
-12 hr	50	.4	80.5 hr	7.0	0.69 (-)
------	-	.4	80.5 hr	7.7	----
-24 hr	8	.4	93.5 hr	5.8	0.12 (+)
-24 hr	50	.4	93.5 hr	5.8	0.12 (+)
-24 hr	-	.4	93.5 hr	5.7	----

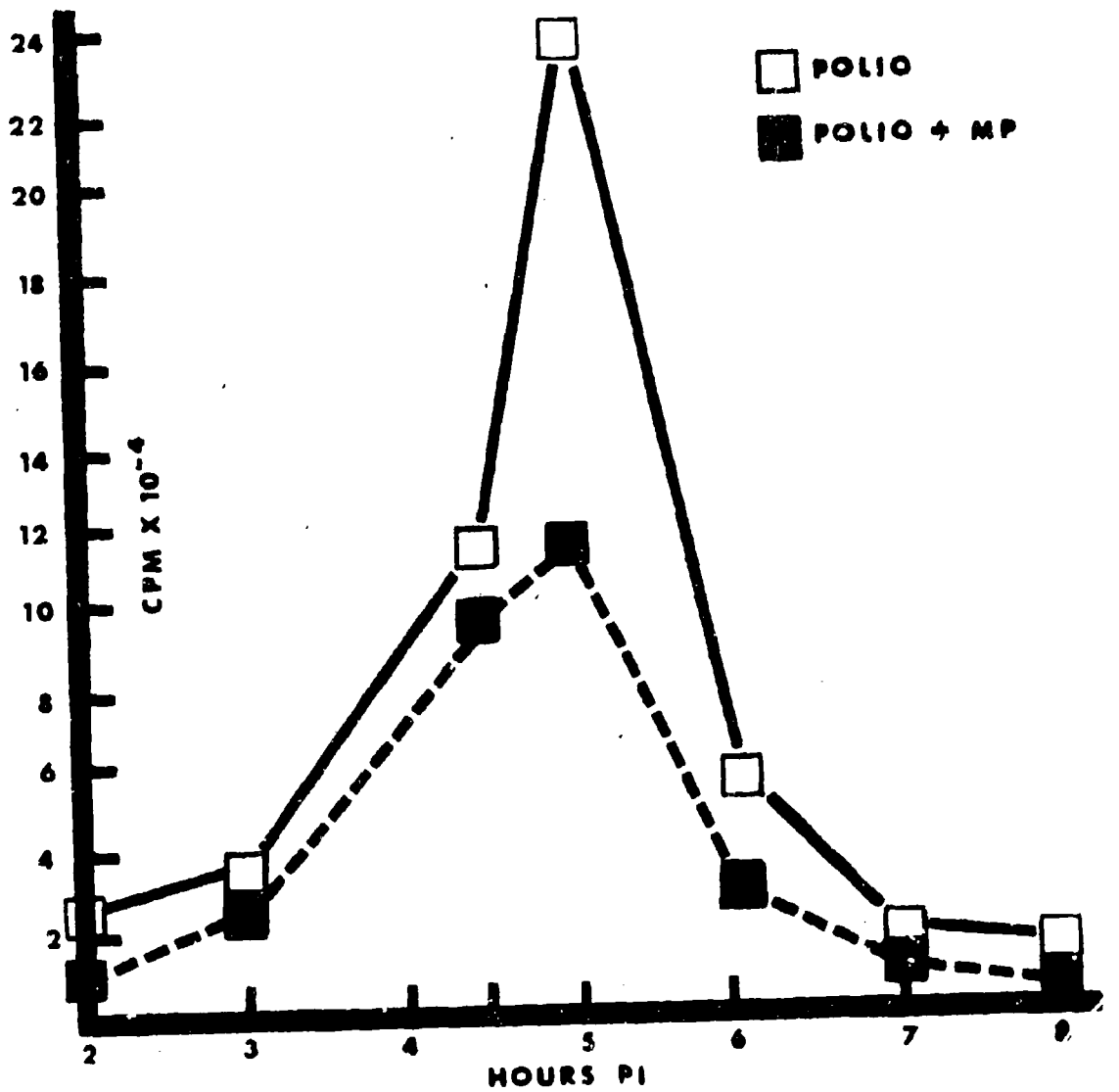
(a) Time of MP: Time of pre-addition of MP in relation to the time of rhinovirus infection.
(b) IM MP: Input multiplicity of inoculation with MP/cell.
(c) IM RV: Input multiplicity of rhinovirus 2060/cell.
(d) Length of incubation: Length of incubation at 33 C on the roller drum following infection with rhinovirus 2060.
(e) Virus yield: Average virus yield for each group (3 tubes/group).

stimulation. There may be inhibition at the greater input multiplicity of mycoplasma. Assuming that it takes 10-11 hours to complete one virus replication cycle, 57.5 hours of incubation would correspond to approximately 5 to 6 viral replication cycles. Cell systems were similarly treated with mycoplasma, and then inoculated with rhinovirus at an input multiplicity of 0.4 $TCID_{50}$/cell. There was no significant change in the virus yield at the lower input multiplicity of mycoplasma, but at the greater input multiplicity there was a decrease of 0.7 $TCID_{50}$/ml in the virus yield. The 80.5 hours required for reaching a $3\text{-}4^{+}$ cytopathic effect in the cell systems inoculated at an input multiplicity of 0.4 $TCID_{50}$/cell corresponds to 7 to 8 replication cycles. Finally, when mycoplasma was inoculated into cell systems 24 hours prior to virus infection, there was no significant change in the virus yields as compared to controls not pre-inoculated with mycoplasma.

In contrast to mycoplasma enhanced rhinovirus-RNA synthesis, polio virus-RNA synthesis was inhibited. M. pneumoniae pre-inoculated cell monolayers were rinsed with maintenance media and infected with polio virus type I at an input multiplicity of 40 PFU/cell. The monolayers were incubated at 37°C and ^{3}H-uridine uptake was measured as previously described. The RNA curve of polio virus in the presence of M. pneumoniae is illustrated in Fig. 2. The viral control showed a RNA curve peaking at 4 to 5 hours post-infection as did the RNA curve of polio in mycoplasma pre-inoculated cells. However, the peak of RNA synthesis in mycoplasma pre-inoculated cells was only half of the control level indicating an inhibitory effect on the RNA-synthesis of polio virus.

Another difference between polio and rhinovirus was observed. Previous studies by Fletcher and Platt (Arch. Ges. Virus forschung 25:1, 1968)

Fig. 2. Inhibition of polio virus-RNA synthesis (^{3}H-uridine uptake) in the presence of M. pneumoniae.



indicated the ability of human gingival cells to replicate rhinovirus. In contrast, polio virus is capable of attaching to human cells of gingival origin, but it was unable to complete its replication cycle as indicated by the absence of viral RNA synthesis.

Occasionally, while assaying for tritiated-uridine incorporation of rhinovirus-infected cells in the presence and absence of mycoplasma, it became evident that stimulation could not be demonstrated. Invariably, these experiments were characterized by the fact that the mycoplasma titer was quite low. To clarify the effect of the titer of the mycoplasma on the degree of stimulation, several experiments were performed. KB cell monolayers were inoculated with various titers of M. pneumoniae 12 hours prior to viral infection, and were then assayed for tritiated-uridine incorporation as before. The results from these experiments indicated that stimulation was observed within certain concentrations of mycoplasma. Input multiplicity of M. pneumoniae in terms of acid-forming units of 8.25 was optimal, with 2.1 or <and 33.0 or> AFU's showing no stimulation or rhinovirus-RNA synthesis. The stimulation is an "all or none" effect depending on the titer of mycoplasma employed.

Previous experiments were all performed with the mycoplasma addition being 12 hours prior to viral infection. Experiments were performed to determine whether the stimulation of viral RNA synthesis could also be observed when cell monolayers were inoculated with mycoplasma at different times prior to rhinovirus infection. Cell monolayers were divided into several groups and were inoculated with identical concentrations of mycoplasma (16 AFU/cell) at 48, 24, 12, and at the time of viral infection. The cells were infected with rhinovirus and assayed for acid-precipitable incorporation. The data from these experiments (Table 5) indicated that

Table 5. Effect of *Mycoplasma pneumoniae* (MP) pre-inoculation at different times prior to rhinovirus infection on the peak of uridine-5-H^3 incorporation into acid-precipitable material.

Time of MP inoculation	Time of Rhinovirus infection	Peak incorporation of uridine-5-H^3
-48	0	76,968 CPM
-24	0	71,929 CPM
-12	0	81,505 CPM
0	0	70,900 CPM
none	0	74,131 CPM

the stimulation was the greatest when mycoplasma was added to the cell monolayers 12 hours prior to viral infection. When mycoplasma was inoculated at 48 and 24 hours prior to viral infection, there was no apparent stimulation.

As stated in the Annual Report Number 1, influenza A/PR8 hemagglutination titers appeared to be enhanced in M. pneumoniae infected Rhesus monkey kidney monolayer systems. In addition, a study was conducted in 1,308 mice to determine if a similar synergistic effect could be demonstrated (Annual Report No. 2). Mice infected with the combination, influenza A/PR8 and M. pneumoniae, have shown: (1) earlier symptoms, (2) earlier deaths and (3) in general, a greater number of deaths, than mice infected with influenza alone. Preliminary trials with germ-free mice show no apparent differences from the results seen in conventional animals. Mycoplasma inoculated at 4, 3, 2 and 1 day prior to the virus infection appeared to produce a similar effect. M. pneumoniae inoculations (without virus) did not produce deaths in mice tested.

Influenza virus, B/Lee, was adapted to replicate in gingival cells after 4 serial passages. No apparent CPE was produced by the virus in gingival cell culture, but hemagglutination titers of 1:32 were obtained by 5 days post-infection at 37°C. Hemadsorption appears to be more sensitive (> 1 log) than the HA test for measuring influenza virus titers. Infectivity titers ($TCID_{50}$/ml) of 10^5 have been obtained in these cultures, of which 0.05 ml (5×10^3 virions) instilled intranasally into NIH mice resulted in 80 percent mortality within 9 days after infection. Influenza virus, replicated in gingival cell culture, was inactivated by ether and pH 2 treatment.

Because adenovirus type 4 is one of the major causative agents of respiratory disease in military recruits it was selected for study in conjunction with M. pneumoniae. These doubly infected systems showed greater DNA synthesis as compared to cells infected with the virus alone (Table 6). However, viral stimulation was observed only when the cell culture systems were treated with mycoplasma at 48 and 24 hours prior to the virus inoculum, (Annual Report No. 2). The positive identity of the labeled DNA (cellular, mycoplasma or virus) was investigated using Sepharose 2B separation. This separation of ^{3}H-thymidine labeled adenovirus type 4 was accomplished at approximately 40 ml of elution volume. M. pneumoniae particles, because of their large size, were eluted in the void volume. The results of these extensive studies showed no conclusive quantitative difference in labeled adenovirus recovered, whether the virus was grown in the presence or absence of mycoplasma.

For the purpose of comparing adenovirus-mycoplasma results with another DNA virus, the herpes simplex virus was selected. Synergistic or antagonistic effects of M. pneumoniae and rhinovirus were tested in combination with these two agents. Adenovirus type 4 pre-treated (minus 24 hours) KB-cell-monolayer systems did not show inhibition of rhinovirus RNA synthesis. In contrast, herpes virus infection 48 and 24 hours prior to rhinovirus inoculation inhibited rhinovirus RNA synthesis by 100% and 90% respectively (Fig. 3).

Infection of KB cells with herpes virus (48 and 24 hours prior to rhinovirus infection) inhibited rhinovirus replication by >99% and 98% respectively (Table 7). Simultaneous infection of the cell systems with a similar herpes-rhinovirus mixture reduced the rhinovirus titer by 94%, whereas the herpes virus titer was completely suppressed. Rhinovirus

Table 6. ^{3}H-Thymidine uptake of adenovirus type 4 in M. pneumonia inoculated and uninoculated L-132 monolayer cell systems.

Adenovirus type 4	*Exposure time to M. pneumoniae (hrs)	COUNTS PER MINUTE									
		TCA Insoluble					TCA Soluble				
		exposure time to thymidine-^{3}H (hours)									
		0-14	14-16	14-18	18-20	18-22	0-14	14-16	14-18	18-20	18-22
+	48	284,602	25,540	36,955	24,705	35,698	15,728	13,393	13,196	12,846	14,786
+	24	227,394	25,607	32,717	23,458	36,991	15,761	10,844	11,878	11,056	14,304
+	6	127,363	18,802	27,498	16,673	23,148	9,727	11,514	12,763	8,807	12,565
+	2	145,335	9,373	17,409	17,940	19,194	9,103	2,281	8,664	10,571	11,762
+	NONE	195,960	17,085	23,435	15,541	24,245	9,346	9,698	13,256	10,114	13,351
0	48	844,670	---	679,137	---	---	19,962	---	56,119	---	---
0	24	776,982	290,924	628,209	414,130	598,223	22,447	86,603	56,000	79,758	27,518
0	6	677,305	312,991	771,282	380,794	524,694	16,150	101,948	82,177	99,737	60,236
0	2	538,532	258,833	689,630	282,002	523,041	19,370	105,240	67,313	78,960	60,988
0	NONE	799,318	289,420	676,407	323,297	549,275	19,335	101,701	74,386	97,338	63,947

*Exposure of L-132 tissue monolayer to M. pneumoniae prior to adenovirus inoculation.

---not tested

Fig. 3. Inhibition of rhinovirus-RNA synthesis (^{3}H-uridine uptake) by Herpes simplex virus in KB cell-monolayer cultures.

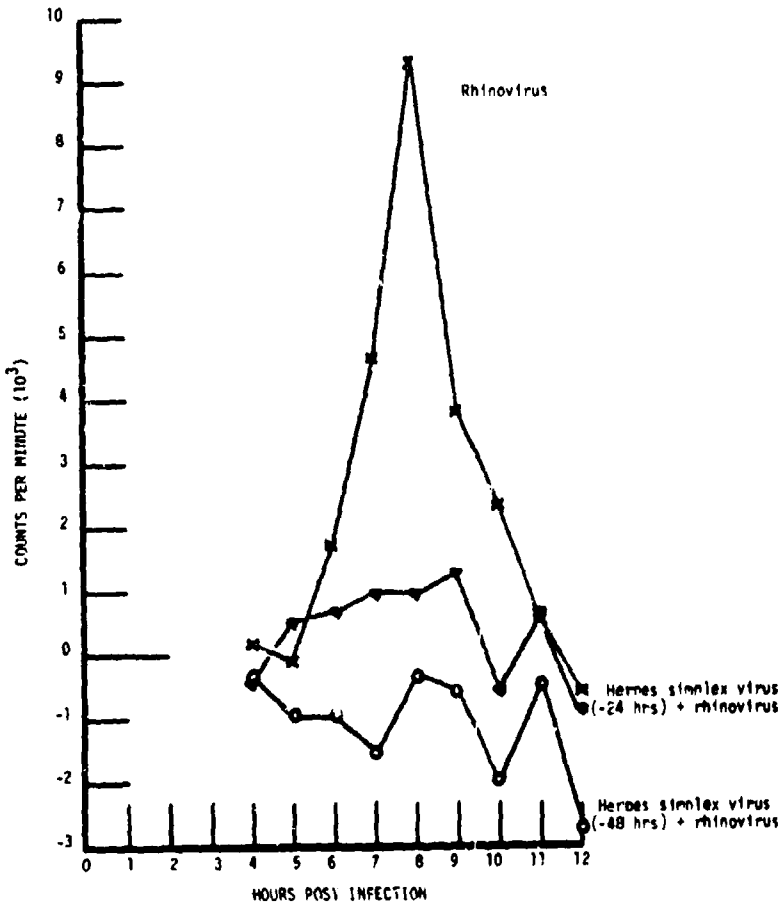


Table 7. Infectivity Titrations of Doubly Infected (Herpes simplex-Rhinovirus) KB Cell Monolayers

Treatment	Herpes Simplex Virus[1]	Rhinovirus[2]	Viral Titer[3]	% Inhibition
Anti-Rhinovirus Serum	48	+	7.7	---
	24	+	5.3	99.60
	0	+	<1	100
	48	-	7.7	---
	--	+	<1	100
Ethyl-Ether	48	+	4.4	99.95
	24	+	6.0	98.00
	0	+	6.5	94.00
	48	-	<1	100
	--	+	7.7	---
None	48	+	5.0	MI[4]
	24	+	4.5	MI
	0	+	5.0	MI
	48	-	6.0	---
	--	+	6.1	---

[1]Time of Herpes simplex virus infections prior to rhinovirus inoculations (input multiplicity, 4)
[2]Rhinovirus inoculations at zero time (input multiplicity, 40)
[3]Log_{10} $TCID_{50}$/ml; average of 3 experiments
[4]MI = mixed infection (Herpes virus and rhinovirus)

infection 24 hours following herpes virus infection inhibited the latter by >99% whereas, rhinovirus infection 48 hours following herpes virus infection resulted in no detectable inhibition. The herpes virus control titers (cell systems infected at 48 and 24 hours) were the same as the value in Table 7.

In contrast, M. pneumoniae added at 48 and 24 hours before herpes simplex virus inoculations appeared to enhance the viral produced CPE in rabbit kidney cell monolayers. The herpes virus yield titered two logs higher in the 48 and 24 hour mycoplasma pre-treated cells than in the cells pre-treated with mycoplasma at minus 6 hours and in the untreated cells.

Because of the sensitivity of M. pneumoniae to actinomycin D, an investigation was conducted to determine the effect on DNA and RNA synthesis (Table 8), and mycoplasma viability. Incorporation of ^{3}H-uridine was inhibited by 73% at a level of 0.019 μg/ml of actinomycin D, whereas there was a 50% reduction of ^{3}H-thymidine uptake at 1.25 μg/ml. Viability of mycoplasma was not effected at levels of 0.625 μg/ml or < up to 2 hours of exposure. Between 2 to 3 hours exposure at 0.625 μg/ml the mycoplasma were completely inactivated.

DISCUSSION

Mycoplasma pneumoniae, as well as components of PPLO medium (PPLO broth, Difco, and fresh yeast extract), stimulate rhinovirus (type 1A, strain 2060)-ribonucleic acid synthesis, but mycoplasma-infected KB cell systems produce virions to the same titer as mycoplasma-free cell systems. Despite this fact, doubly infected cells show qualitatively a more extensive cytopathic effect and an earlier CPE was observed than in viral

Table 8. Effect of actinomycin D on ^{3}H-uridine and ^{3}H-thymidine uptake of *Mycoplasma pneumoniae* monolayers.

Actinomycin D[a] µg/ml	Counts/Minute	
	^{3}H-thymidine[b]	^{3}H-uridine[c]
NONE	18,685	3336
0.019	18,216	894[d]
0.039	16,479	700
0.078	15,908	625
0.156	13,756	479
0.312	13,178	355
0.625	10,471	286
1.25	9,641[d]	372
2.5	7,799	332
5.0	4,042	233
10.0	1,209	162
20.0	565	141

a. actinomycin D was added to the *M. pneumoniae* monolayers 1 hr prior to the radioactive label.
b. ^{3}H-thymidine 4µc/ml, 1 hr pulse (average of 3 experiments)
c. ^{3}H-uridine 2.5µc/ml, 1 hr pulse (average of 7 experiments)
d. 50% or > inhibition

infected mycoplasma-free cells. In vivo this might account for a more severe infection when these two respiratory agents are involved.

Concerning stimulation of rhinovirus-RNA synthesis by constituents of PPLO growth medium, yeast extract accounted for the highest level of enhancement. Yeast extract has frequently been shown by others to enhance bacterial growth, however, stimulation of viral replication is unique.

In contrast to this enhancing effect in the presence of M. pneumoniae, polio virus-RNA synthesis was inhibited. This would indicate the specificity of this type of interaction where even members of the same group of viruses react differently. The mechanism-of-action of this mycoplasma produced effect on the host cell's ability to replicate viruses is not clear. There has been some indication that the host cell membrane permeability has been changed by the mycoplasma resulting in increased penetration rates of rhinovirus. It has been demonstrated that mycoplasma are not capable of multiplying in cell culture medium and this would indicate that the mycoplasma replicate in association with the cell, usually the cell membrane (Clyde, Am. Rev. Resp. Dis. 88:212, 1963). M. pneumoniae altered the growth curve of KB cells. The cells replicated more slowly and reached concentrations 24% less than if the cells were grown in the absence of mycoplasma. This altered growth curve of cells in the presence of M. pneumoniae may be due to the production of hydrogen peroxide or depletion of nutrients from the culture medium or a combination of both. However, it doesn't seem likely that this could explain the inhibition of polio virus and enhancement of rhinovirus-RNA synthesis.

When the pattern of RNA synthesis in rhinovirus-infected KB cells was examined, some interesting facts became apparent. Replication of rhinovirus RNA in KB cells began between 3 to 4 hours post infection with a

peak at 8 to 9 hours post-infection. Efficiency of incorporation of ^{3}H-uridine into acid-precipitable material was proportional to the input multiplicity of infection e.g. input multiplicities of 40, 4, and 0.4 $TCID_{50}$/cell, 96%, 68% and 46% respectively, of the cells were infected on the basis of trypan blue. Therefore, the difference in the patterns of RNA synthesis at the varying input multiplicities were most probably due to the fact that at the lower input multiplicities few virions attached to the cells and initiated infection.

In the experiments described above, influenza virus inoculations were conducted in ether anesthetized mice. The possibility exists that some of the mycoplasma inoculum may have been inactivated by the ether, however, the effect of the viral infection was still enhanced by the mycoplasma addition. Clyde (Yale. J. Biol. Med. 40:436, 1968) has suggested using sodium secobarbitol to anesthetize laboratory animals thus preventing the possibility of ether inactivation of the mycoplasma. In this respect, influenza viruses are also inactivated by ether, yet the ether anesthesia technique described above did not appear to inactivate the virus.

Gingival cells appear to have receptor sites specific for attachment of influenza virus and are able to propagate viable virions. There may be some value in using gingival cells, instead of more commonly used cell cultures for virus propagation.

Adenovirus-mycoplasma interactions were hampered by the lack of a suitable inhibitor to suppress host cell-mycoplasma nucleic acid synthesis while allowing viral replication. As a result, host cells were not inhibited except after adenovirus infection, e.g. host-cell DNA is inhibited by adenovirus type 5 at approximately 10 hours post-infection,

whereas viral DNA synthesis occurs between 10 and 21 hours post-infection (Ginsberg, Bello, and Levine, in the Molecular Biology of Viruses (Colter and Paranchych, eds), Academic Press, N.Y., 1967, p. 547). In adenovirus-mycoplasma studies ^{3}H-thymidine was added after host DNA synthesis was predicted to be inhibited. Tissue controls and mycoplasma-treated cell systems showed high thymidine incorporation compared to the lower levels of DNA synthesis observed in the mycoplasma-virus and virus infected systems. In general, the mycoplasma-adenovirus incorporation of thymidine was higher than in the virus infected systems (no mycoplasma treatment). The increased DNA synthesized was probably viral, as supported by the fact viral endpoint titers in KB or L132 cells were greater in the presence of mycoplasma. In addition, great numbers of virus infected cells were observed in mycoplasma-cell cultures, compared to untreated cells, as demonstrated by fluorescent antibody techniques.

Reduced replication of herpes simplex virus has been demonstrated in myxovirus-infected cultures (Szanto and Lesso, Acta Virol. 15:47, 1971). In these doubly-infected systems a single cell contained detectable antigen for only one virus. In contrast to aforementioned viral interference, herpes simplex virus provided incomplete helper functions for the potentiation of defective adeno-associated virus (Blacklow, et al. PSEBM 134:952, 1970). These results suggested the possibility that AAV may participate in the disease response to herpes virus as well as to adenovirus infections. Because herpes virus had the potential of being an active interfering agent or provide a helper function, it's effect on rhinovirus replication was tested. In these investigations, infection of KB cells with herpes simplex virus prior to rhinovirus infection resulted in inhibition of rhinovirus replication, as was demonstrated by reduction

in both rhinovirus yield and ^{3}H-uridine uptake. The viral interference phenomenon occurred in both directions, i.e. rhinovirus can also inhibit herpes simplex virus but in a limited period (up to 24 hours) post-herpes infection. The two viruses when inoculated simultaneously produced strong interference to each other and resulted in reduction of the yield of both viruses (Table 7). A possible explanation for this observed viral interference would be 1) viral produced biochemical changes in the host cells or 2) interferon production.

Finally, actinomycin D is extremely effective in inhibiting mycoplasma RNA synthesis, and clearly indicates the ideal nature of actinomycin D utilization in mycoplasma-rhinovirus-cell system for inhibiting all but viral-RNA synthesis.

CONCLUSIONS

1. An ideal system for measuring the effect of mycoplasma on rhinovirus (and other select RNA viruses) RNA synthesis was established using ^{3}H-uridine uptake in actinomycin D-treated cell systems.

2. M. pneumoniae significantly enhances rhinovirus-RNA synthesis in KB cell monolayers (M. pneumoniae added 12 hours prior to the virus inoculum) (M. pneumoniae input number per cell was optimally 8).

3. M. pneumoniae pre-inoculated cell subsequently infected with rhinovirus has no effect on the final virus yields under conditions where there was stimulation of viral RNA synthesis.

4. Polio virus-RNA synthesis was inhibited by mycoplasma in pre-inoculated cells.

5. The only meaningful data to be obtained from rhinovirus uptake of ^{3}H-uridine in actinomycin D treated cell systems was that of acid-

precipitable and acid soluble incorporation, indicating RNA synthesis and phosphorylation respectively.

6. The most efficient growth cycle occurred if cell monolayers were infected with rhinovirus at an input multiplicity of 40 $TCID_{50}$/cell (96% of cells infected). At an MOI of 4 and 0.4 only 68 and 46% of the cells were infected.

7. M. pneumoniae showed little effect on KB cell viability until 124 hours post-inoculation, when there was a gradual decrease in the viable cells (38.4% reduction).

8. Influenza virus, A/PR8, infections in mice appeared to be enhanced by M. pneumoniae based on earlier symptoms, earlier deaths and a greater number of deaths in the doubly infected animals.

9. Adenovirus-M. pneumoniae infected L-132 cells showed greater DNA synthesis (M. pneumoniae additions 24 and 48 hours prior to the virus inoculum).

10. Herpes simplex virus and rhinovirus infections of KB cells showed that the viral interference phenomenon occurred in both directions but the herpes virus was the more sensitive.

11. M. pneumoniae pre-treatment (minus 48 and minus 24 hours) of rabbit kidney cells stimulated herpes simplex virus yields by two logs higher than the titer of herpes virus grown in mycoplasma-free cells.

12. Actinomycin D treatment of M. pneumoniae inhibited ^{3}H-uridine uptake by 90% at 0.312 μg/ml and ^{3}H-thymidine uptake by 90% between 5 and 10 μg/ml.

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