AD-754 657

BASIC STUDIES ON INTERACTIONS OF AGENTS CAUSING RESPIRATORY INFECTIONS

Ronald D. Fletcher

Pittsburgh University

Prepared for:

Army Research Office

15 January 1973

AD_____

REPORT NUMBER 1

BASIC STUDIES ON INTERACTIONS OF AGENTS CAUSING RESPIRATORY INFECTIONS

FINAL REPORT

By

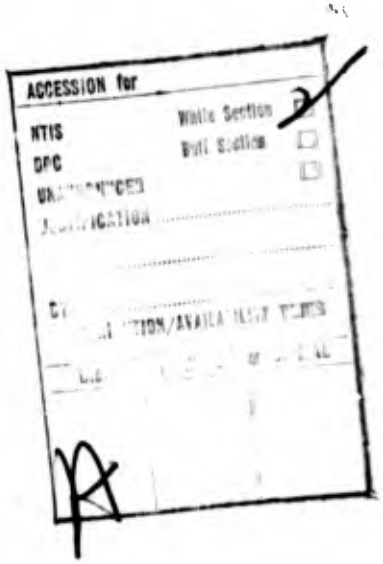
Ronald D. Fletcher, Ph.D.

Department of Microbiology
School of Dental Medicine
University of Pittsburgh
Pittsburgh, Pa. 15213

January 1973

Life Sciences Division
Army Research Office
3045 Columbia Pike
Arlington, Virginia 22204

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.



Security Classification

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author)

Ronald D. Fletcher
Department of Microbiology
School of Dental Medicine
University of Pittsburgh
Pittsburgh, Pa. 15213

2a. REPORT SECURITY CLASSIFICATION: Unclassified

2b. GROUP:

3. REPORT TITLE

Basic Studies on Interactions of Agents Causing Respiratory Infections

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)

Final Report: January 1, 1972 to December 31, 1972

5. AUTHOR(S) (First name, middle initial, last name)

Same as in no. 1.

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
January 15, 1973	38	11

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
DAHC 19-72-G-0007	DAHC 19-72-G-0007
b. PROJECT NO.	
c.	9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
d.	

10. DISTRIBUTION STATEMENT

Unlimited

11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
	Life Sciences Div. Army Research Office 3045 Columbia Pike Arlington, Virginia 22204

13. ABSTRACT

The importance of respiratory infections, especially the probable interaction of respiratory pathogens, in military populations was the basis of this investigation. It was hoped to establish synergistic, additive or inhibitory effects on replication, nucleic acid synthesis or survival of selected respiratory viruses (e.g., rhinovirus and influenza virus) and M. pneumoniae.

Prior to this report, it had been demonstrated by Milligan and Fletcher, 1969 that M. pneumoniae enhanced rhinovirus 1A/2060-RNA synthesis. During this report period another rhinovirus (16/11757) was studied under similar conditions and also showed enhanced viral-RNA synthesis in M. pneumoniae infected cells. Despite mycoplasma stimulation of nucleic acid synthesis viral titers remained at the same level as those in virus systems not exposed to mycoplasma.

To demonstrate any close relationship between rhinovirus 1A/2060 and M. pneumoniae and M. orale, electron microscopy studies were conducted. These electron micrographs showed no virus attached to mycoplasma or intracellular rhinovirus crystals in the M. pneumoniae or M. orale cells.

The electron micrographs may not have indicated some molecular level effect of rhinovirus on mycoplasma, therefore a study was conducted to determine the effect of these viruses on ^{3}H-thymidine uptake of M. pneumoniae. This investigation showed viable virus as well as heat inactivated virus resulted in decreased ^{3}H-thymidine uptake. The significance of this finding is unknown, but virus preparations exposed to ^{3}H-thymidine did not seem to adsorb sufficient radioactive isotope to account for this loss. In contrast to these findings, actinomycin D (5µg/ml) resulted in 84% to

DD FORM 1 NOV 68 1473 REPLACES DD FORM 1473, 1 JAN 64, WHICH IS OBSOLETE FOR ARMY USE.

I

Security Classification

14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
96% inhibition of M. pneumoniae uptake of ^{3}H-uridine. Further studies to detect a protective effect of mycoplasma on viruses (rhinovirus and influenza) was conducted. Rhinovirus had a similar survival rate at 37°C in the presence or absence of mycoplasma without mammalian cells. Influenza virus was partially inactivated at 60 min exposure to M. pneumoniae (HA titer, log=1.8), compared to the control without mycoplasma (HA titer, log=2.3). At 120 mins exposure both systems had a decrease in viral titer.						

Security Classification

AD_____

REPORT NUMBER 1

BASIC STUDIES ON INTERACTIONS OF AGENTS CAUSING RESPIRATORY INFECTIONS

FINAL REPORT

By

Ronald D. Fletcher, Ph.D.

Department of Microbiology
School of Dental Medicine
University of Pittsburgh
Pittsburgh, Pa. 15213

Grant No. DAHC 19-72-G-0007
January 1, 1972 through December 31, 1972

January 15, 1973

Life Sciences Division
Army Research Office
3045 Columbia Pike
Arlington, Virginia 22204

TABLE OF CONTENTS

SUMMARY

The importance of respiratory infections, especially the probable interaction of respiratory pathogens, in military populations was the basis of this investigation. It was hoped to establish synergistic, additive or inhibitory effects on replication, nucleic acid synthesis or survival of selected respiratory viruses (e.g., rhinovirus and influenza virus) and M. pneumoniae.

Prior to this report, it had been demonstrated by Milligan and Fletcher, 1969, that M. pneumoniae enhanced rhinovirus 1A/2060-RNA synthesis. During this report period another rhinovirus (16/11,757) was studied under similar conditions and also showed enhanced viral-RNA synthesis in M. pneumoniae infected cells. Despite mycoplasma stimulation of nucleic acid synthesis viral titers remained at the same level as those in virus systems not exposed to mycoplasma.

To demonstrate any close relationship between rhinovirus 1A/2060 and M. pneumoniae and M. orale, electron microscopy studies were conducted. These electron micrographs showed no virus attached to mycoplasma or intracellular rhinovirus crystals in the M. pneumoniae or M. orale cells.

The electron micrographs may not have indicated some molecular level effect of rhinovirus on mycoplasma, therefore a study was conducted to determine the effect of these viruses on ^{3}H-thymidine uptake of M. pneumoniae. This investigation showed viable virus, as well as heat inactivated virus, resulted in decreased ^{3}H-thymidine uptake. The significance of this finding is unknown, but virus preparations exposed to ^{3}H-thymidine did not seem to adsorb sufficient radioactive isotope to

account for this loss. In contrast to these findings, actinomycin D (5μg/ml) resulted in 84% to 96% inhibition of M. pneumoniae uptake of ^{3}H-uridine.

Further studies to detect a protective effect of mycoplasma on viruses (rhinovirus and influenza) was conducted. Rhinovirus had a similar survival rate at 37°C in the presence or absence of mycoplasma. Influenza virus was partially inactivated at 60 min exposure to M. pneumoniae (HA titer, log=1.8), compared to the control without mycoplasma (HA titer, log=2.3). At 120 mins exposure both systems had a decrease in viral titer.

INTRODUCTION

Respiratory diseases due to viruses and certain mycoplasma are an ever present problem for the military population resulting in the loss of millions of man-hours per year. In some instances these agents are found in combinations that might result in a more severe infection. Because of this possibility respiratory viruses, e.g. rhinovirus and influenza virus, were interacted with Mycoplasma pneumoniae to determine enhancement or inhibition of replication and nucleic acid synthesis. Information on the following was collected: effect of M. pneumoniae on rhinovirus RNA synthesis, thymidine uptake of M. pneumoniae as affected by actinomycin D and rhinoviruses, rhinovirus and polio yields in mycoplasma-infected cell systems, electron microscopy of mycoplasma-virus associations, and survival rates of influenza virus exposed to mycoplasma.

MATERIALS AND METHODS

Respiratory Agents: Rhinovirus type 1A (strain 2060), type 12 (strain 181-CY16) and type 16 (strain 11, 757), propagated in HeLa or KB cells, were obtained from the American Type Culture Collection (ATCC). Polio virus type 1, propagated in HeLa or KB cells, was also procured from ATCC. Influenza virus A2/Hong Kong (propagated in canine kidney cells) was obtained from Dr. J. Youngner, U. of Pittsburgh, Pittsburgh, Pa. M. pneumoniae and M. orale were obtained from the ATCC. Mycoplasma were grown in PPLO growth medium (Difco PPLO broth, 20% agamma horse serum, 10% fresh yeast extract and 0.5% glucose).

Viral RNA Synthesis: Procedure for measuring rhinovirus incorporation of ^{3}H-uridine was described by Fletcher, 1969.

Electron Microscopy: An effort was made to demonstrate possible rhinovirus replication in Mycoplasma pneumoniae cells grown on glass and M. orale grown in suspension culture in 16 x 125mm screw cap tubes. This study was encouraged by electron micrographs of intracellular rhinovirus crystals in M. orale type 1 cells (Fiala, personal communication, 1971).

Rhinovirus inoculated M. pneumoniae and M. orale monolayers were incubated for 1, 2, 4, 8, 24 and 48 hours and then pelleted by slow speed centrifugation. For the electron microscopic studies, the pellets were treated with cold (0-5°) 4.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.3 for 30 minutes (Sabatini, D.D., Bensch, K., and Barnett, R.J., J. Cell Biol. 17, 19-58, 1963). The packed cells were washed in several changes of buffer, dehydrated in alcohol and propylene oxide, and embedded in Epon 812 (Luft, J.H., J. Cell Biol., 9, 409-414, 1961). Silver to light gold sections were cut on a Porter-Blum MT-2 ultramicrotome, stained in uranyl acetate and lead citrate (Reynolds, E., J. Cell Biol. 17, 208-212, 1963) and examined in a Philips EM-200 electron microscope at original magnifications of 50,000-200.000 diameters.

Virus Survival on M. pneumoniae Exposure: Survival rate of rhinovirus 12/181 and Influenza A2/Hong Kong were studied in the presence and absence of M. pneumoniae. These mycoplasma monolayers contained in screwcap tubes (16 x 125mm) were drained, washed 3x with 2 ml volumes of Earle's cell culture medium (BME), drained and 1 ml of rhinovirus stock was added to the mycoplasma culture. One ml of rhinovirus stock was also added to screwcap tubes (16 x 125mm) containing no mycoplasma cells. All tubes were incubated at 33°C, and after 24 hrs and 48 hrs tubes from each

group were frozen at -60°C. The mycoplasma-virus systems and the virus systems were thawed, frozen for a second time, thawed and the virus titered in KB cell monolayers.

Effect of Rhinovirus on Mycoplasma ^{3}H-Thymidine Uptake: Effect of rhinovirus on M. pneumoniae monolayers was measured by ^{3}H-thymidine uptake (Table 2). The M. pneumoniae monolayers were grown in PPLO growth medium contained in 16 x 125mm screwcap tubes. When the mycoplasma cell monolayers were confluent they were drained, inoculated with 0.5 ml of virus stock and 0.5 ml of ^{3}H-thymidine (final concentration of 5μc/ml); incubated at 37°C for 4 and 24 hours at which time the mycoplasma sheets were drained, washed 3x with 2 ml portions of TCA, drained and solubilized with 1 ml hyamine and 10 ml scintillation fluid. This procedure is similar to the one described for measuring viral-RNA synthesis.

Because of the possibility the virus may adsorb ^{3}H-thymidine, virus stock was mixed with ^{3}H-thymidine 5μc/ml incubated for 1 and 8 hours at 37°C and centrifuged at 28,000 rpm in the 30 rotor of a Spinco model L ultracentrifuge for 4 hr at 4°C. The supernatant fluid was measured for radioactivity in Triton-X Scintillation fluid and counted in a Packard Tricarb Spectrophotometer.

Effect of Actinomycin D on ^{3}H-uridine uptake by Mycoplasma: This procedure is described below, Fig. 2.

RESULTS

Previously, Fletcher, Milligan and Albertson reported that rhinovirus 1A/2060-RNA synthesis was stimulated by M. pneumoniae. The question

existed as to whether other rhinoviruses would show a similar effect. Because rhinovirus 1A/2060 is a designated M strain, a H strain, rhinovirus 16/11,757 was selected for study. This virus in actinomycin treated KB cells previously infected with M. pneumoniae (21.5 hours before viral inoculation) also showed greater ^{3}H-uridine uptake (Fig. 1). The mycoplasma stimulation of viral-RNA synthesis appeared similar in the presence and absence of PPLO growth medium and peaked at 8 hours post viral infection. A second peak of virus (no exposure to mycoplasma) and virus-mycoplasma (no PPLO growth medium) was also seen at 6 hours. The significance of these two peaks of ^{3}H-uridine uptake are not known.

Rhinovirus added to KB cells chronically infected with M. pneumoniae did not alter the virus yield (Table 1). At several passage levels the chronically infected KB cells were tested for their ability to support rhinovirus growth. Microscopically, the chronically-infected KB cells appeared normal with the exception of a slight granularity. Table 1 shows that M. pneumoniae titers in KB cells were as great as 10^{4} CFU/ml. However, rhinovirus yields were not altered by the presence of the mycoplasma. In KB cell monolayers which were mycoplasma-free as well as in the chronically-infected KB cells, rhinovirus yields were approximately 20 $TCID_{50}$/cell. Therefore, KB cells chronically infected with M. pneumoniae supported the replication of rhinovirus similar to control cell systems, despite the presence of high titers of mycoplasma. In comparison, polio virus yields did not appear to be affected by M. pneumoniae despite inhibition of poliovirus-RNA synthesis (Fletcher, 1971).

In an effort to determine if mycoplasma attach to or penetrate into mycoplasma cells, electron microscopy was conducted as described above.

Fig. 1. Enhancement of rhinovirus 11,757-RNA synthesis (^{3}H-uridine uptake) by Mycoplasma pneumoniae and M. pneumoniae plus PPLO medium.

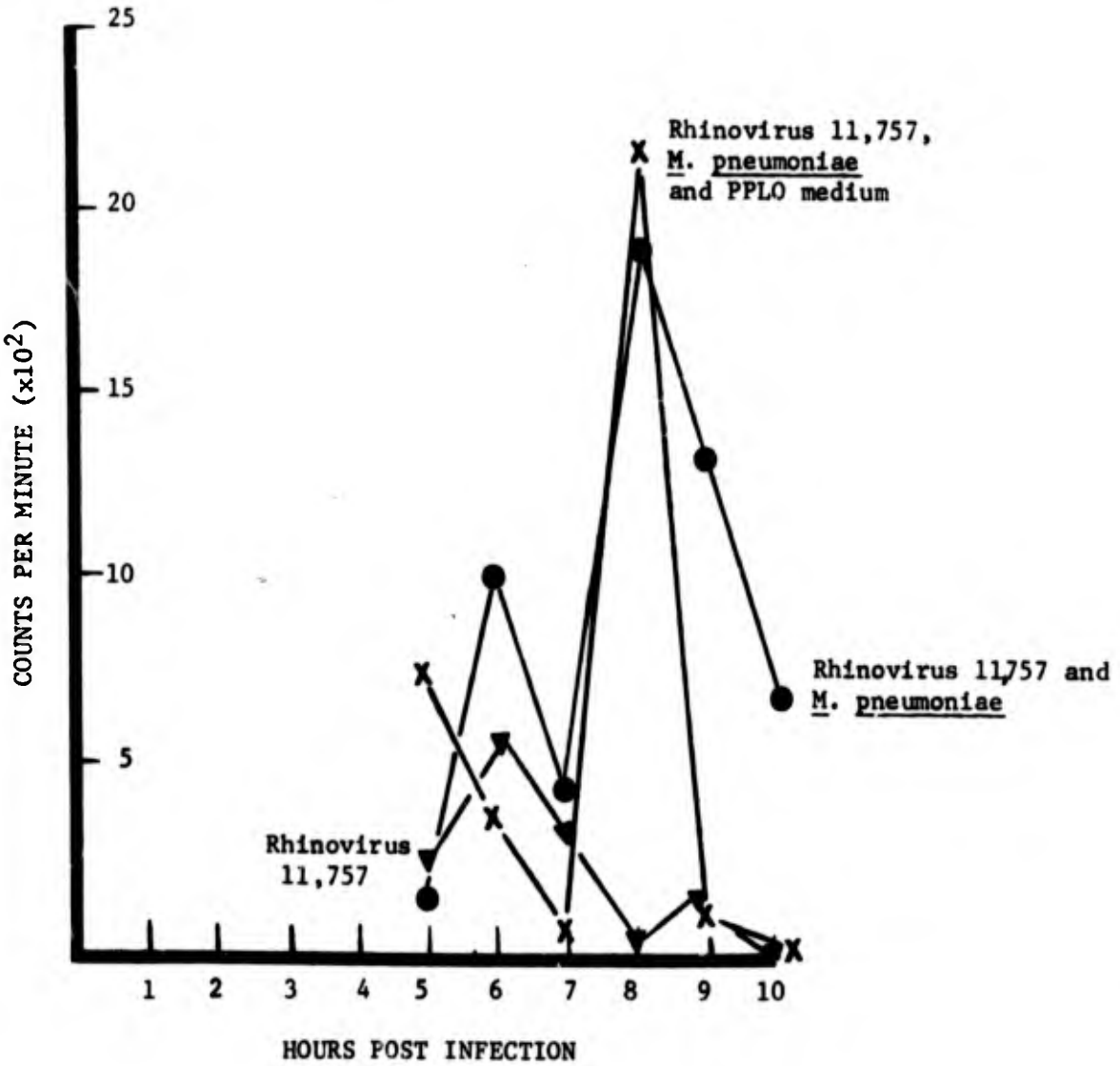


TABLE 1. Rhinovirus Yields in KB Cells Chronically Infected with M. pneumoniae(a). Input Multiplicity of Rhinovirus ($TCID_{50}$/cell)=40.

KB Cells, Passage Number(b)	Titer M. pneumoniae(c)	Virus Yield(d) ($TCID_{50}$/Cell)
-	-	20
1	4.6×10^4	21.3
2	4.3×10^4	20.7
3	3.9×10^4	22.3
4	4.5×10^4	23.1
6	5.0×10^4	20.5
10	4.7×10^4	19.8
13	4.8×10^4	20.1

(a) KB cell monolayers initially inoculated with 4×10^6 acid-forming units (AFU) of M. pneumoniae grown on glass, and incubated at 37C.

(b) Passage number of chronically infected KB cells.

(c) Titer of M. pneumoniae present in the supernatant fluid of chronically infected KB cells at the time of viral infection.

(d) The virus yield represents cell associated and released virus titers.

These electron micrographs showed neither an indication of intracellular rhinovirus crystals in the M. pneumoniae or M. orale cells, nor did they indicate any viral attachment to or penetration into the mycoplasma. However, both virus and mycoplasma were detected in association with each other.

Continuation of earlier studies of the effect of actinomycin D on ^{3}H-uridine uptake of M. pneumoniae showed the level of incorporation of ^{3}H-uridine into TCA-insoluble material of actinomycin D-treated M. pneumoniae monolayers was constant throughout the experimental period. Incorporation of ^{3}H-uridine into TCA-insoluble material of M. pneumoniae monolayers was inhibited 84% to 96% by actinomycin D (Fig. 2). In control monolayers, the level of ^{3}H-uridine incorporation into TCA-insoluble material increased from 2 through 9 hours and then reached a plateau. Detachment of M. pneumoniae from the glass was minimal and was the same in both actinomycin D inoculated and control systems.

^{3}H-thymidine uptake of M. pneumoniae monolayers inoculated with rhinoviruses was investigated (Table 2). The viable virus, as well as the heat inactivated virus, resulted in decreased ^{3}H-thymidine uptake. The significance of this finding is not known, but it would appear that some component of the virus or the virus-conditioned cell culture medium induces this change.

Because of the possibility the virus preparation may adsorb this compound, virus was exposed to ^{3}H-thymidine for 1 and 8 hours, centrifuged and the supernatant fluid measured for radioactive isotope. The virus system supernatant fluid accounted for 93.3% and 102.3% of the control

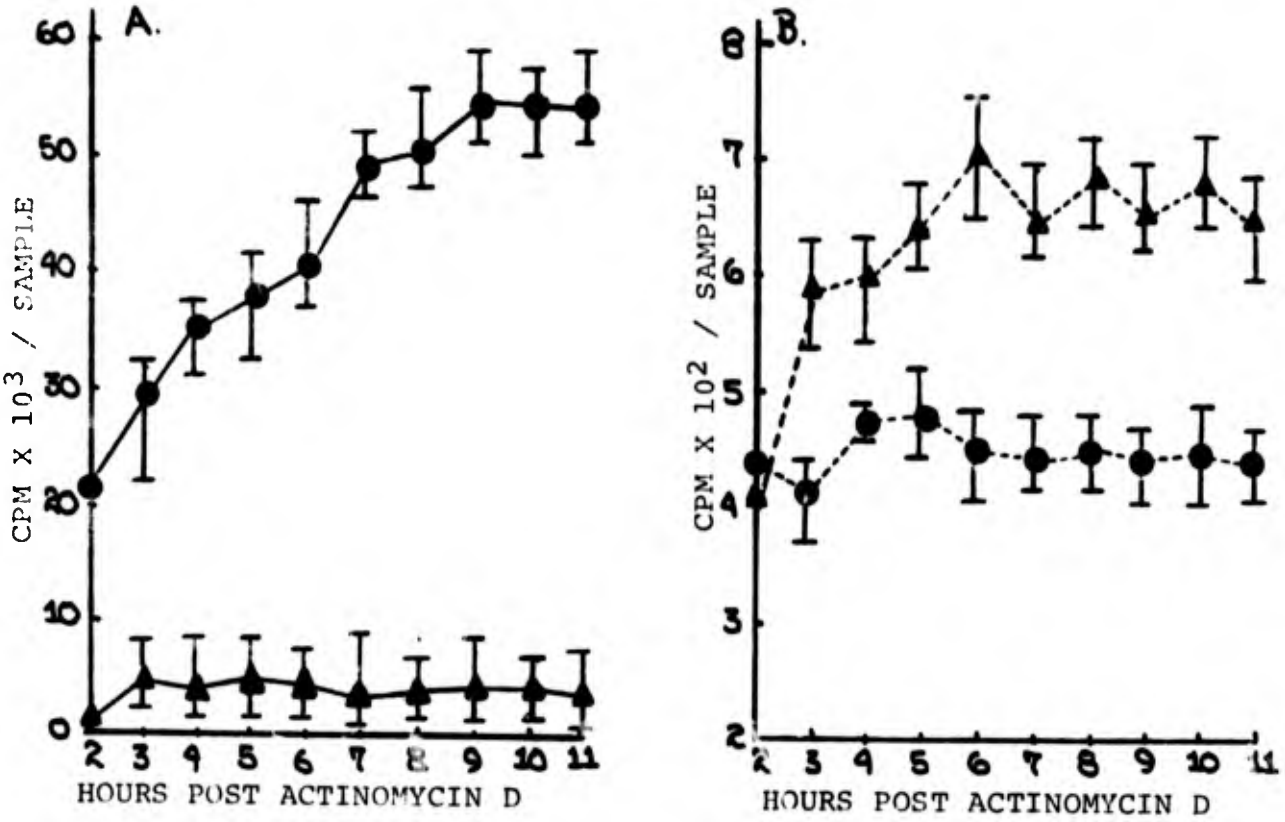


Fig. 2. Effect of actinomycin D on uridine incorporation by M. pneumoniae. MP-G monolayers (10^7-10^8 AFU/tube) in screw-cap tubes were inoculated with actinomycin D (final concentration, 5 ug/ml) or MM and incubated in a roller drum at 33° C. Following a 60 minute incubation period, uridine-5-H^3 was added (final concentration, 4 uc/ml) and incubation was continued. Triplicate samples were removed and monitored for TCA-insoluble (Panel A) and TCA-soluble (Panel B) incorporation.

Symbols: (▲), MP-G monolayers treated with actinomycin D; (●), MP-G monolayers sham-inoculated with MM; (I), range. The data represent the averaged results of three separate experiments.

TABLE 2. ^{3}H-Thymidine Uptake of M. pneumoniae Monolayers Inoculated with Rhinovirus

Inoculum[a]	CPM[c]	
	Hours post-virus inoculation	
	4	24
Rhinovirus 12/181	30,241	52,119
Rhinovirus 12/181[b]	30,687	54,176
Rhinovirus 2060	27,456	50,437
NONE	52,304	90,197

a. 0.5 ml of virus stock
b. Heat inactivated, 56°C 10 mins.
c. TCA precipitate

systems (no virus, cell culture maintenance medium with 2% calf serum), at 1 and 8 hours respectively.

Survival of rhinovirus 1A/2060 and influenza virus A2/Hong Kong exposed to M. pneumoniae and titered in KB cells and canine kidney cells, respectively, was investigated. This study showed no protective effect by M. pneumoniae on rhinovirus survival. Rhinovirus exposed to mycoplasma and rhinovirus alone were both inactivated by 1 log within 48 hours at 33°C and rhinovirus exposed to mycoplasma was inactivated by 6 logs between 3 and 4 days. Similar studies with influenza virus, A/2 Hong Kong showed partial viral inactivation by exposure to M. pneumoniae (Table 3). Because both of these viruses are inactivated at a low pH, it is possible that acid production of M. pneumoniae results in virus inactivation.

DISCUSSION

In this study, the interaction of virus and mycoplasma have been measured by the above mentioned methods. This data showed M. pneumoniae enhancement of rhinovirus 1A/2060 and 16/11,757 RNA synthesis, whereas poliovirus RNA synthesis was inhibited (Fletcher, 1971). Despite this effect on ^{3}H-uridine uptake (viral-RNA synthesis) the replication yields (viable virus) of both agents was similar to virus control systems not exposed to mycoplasma. It would appear that excess viral nucleic acid is produced normally and that inhibition or enhancement of <50% of the total RNA synthesized was not expressed in the number of viable virus.

It appeared rhinovirus was exerting an effect on the mycoplasma cells as suggested by Fiala (personal communication), who observed intra-

Table 3. Survival of Influenza A2/Hong Kong exposed to Mycoplasma pneumoniae monolayers, and titered in canine kidney monolayers.

Exposure[a] Time (Mins)	HA Titer (Log)[b]	
	M. pneumoniae treatment	No Treatment
5	1.8	2.7
15	1.7	2.6
30	1.8	2.6
60	1.8	2.3
120	1.7	1.4

[a]exposed to M. pneumoniae monolayers or allowed to incubate with exposure for the above indicated times at 37°C before inoculation into canine kidney monolayer systems.

[b]Mean value of results of triplicate tests.

cellular rhinovirus crystals in M. orale type 1. However, in this study rhinovirus was not observed to attach or penetrate the host cell. The virus did appear to reduce the ability of the mycoplasma to incorporate ^{3}H-thymidine. Heat-inactivated virus had the same effect, yet the viral preparations did not appear to adsorb ^{3}H-thymidine.

In this regard ^{3}H-thymidine (Fletcher, 1971) and ^{3}H-uridine uptake by M. pneumoniae was inhibited by actinomycin D. The levels of this chemical required for inhibition were far below those concentrations reported by Tourtellotte, (1969).

Finally, interaction of microorganisms can prevent disease, group A streptococci inhibition by normal oral flora (Crowe et al., 1972), or produce disease, as is the case with Corynebacterium diphtheriae, that are lysogenic for prophage-B (Freeman, 1951). Interactions with respiratory agents are of great interest, but they are difficult to establish even with adequate control systems. This is especially true between bacteria and viruses.

CONCLUSIONS

1. Rhinovirus 16/11,757 - RNA synthesis was enhanced by M. pneumoniae in KB cell systems.

2. Rhinovirus and polio virus harvested from M. pneumoniae infected cells showed similar concentrations of viable virus as compared to virus control systems (no mycoplasma exposure).

3. Actinomycin D inhibited ^{3}H-uridine uptake of M. pneumoniae and viable and inactivated rhinovirus appeared to effect M. pneumoniae incorporation of ^{3}H-thymidine.

4. Survival time of influenza virus A2/Hong Kong in combination with M. pneumoniae was decreased.

5. Electron micrographs of rhinovirus - mycoplasma combinations showed neither attachment between these agents nor intracellular viral crystals.

REPORTS AND PUBLICATIONS

The following publications apply to this study and are listed for the convenience of the reader. Previous reports were supported by grant number DAHC 19-69-G-0011 (January 1, 1969) through DAHC 19-71-G-0009 (December 31, 1971), and current reports were supported by grant number DAHC 19-72-G-0007 (January 1, 1972 through December 31, 1972).

Previous Reports or Publications:

Fletcher, R.D. 1969, 1970 and 1971. The Relationship Between Mycoplasma Species and Selected Respiratory Viruses (Adenovirus, Influenza Virus and Rhinovirus). Annual Report Number 1, 2 and 3, Life Science Division, Army Research Office, Arlington, Virginia, 22204.

Milligan III, W.H. and R.D. Fletcher. 1969. The effect of Mycoplasma pneumoniae on rhinovirus-RNA synthesis in KB cells. Antimicrobial Agents and Chemotherapy-1969: 196-199.

Fletcher, R.D., W.H. Milligan III and J.N. Albertson, Jr. 1969. Contributing factors to Mycoplasma pneumoniae produced stimulation of rhinovirus-RNA synthesis. Bull. Czech. Soc. for Microbiology, 5:34 (Abstract).

Fletcher, R.D. and R.A. Johnson. 1970. DNA synthesis of mycoplasma in human gingival cell culture. Internat. Assoc. for Dental Research, p. 87.

Fletcher, R.D., W.H. Milligan III and J.N. Albertson, Jr. 1970. Contributing factors to Mycoplasma pneumoniae on rhinovirus-RNA synthesis in KB cells. Folia Microbiol., 15(5):325-323.

Fletcher, R.D. and R.A. Johnson. 1971. Interaction of Respiratory Agents and Herpes Simplex Virus In Vitro. Internat. Assoc. for Dental Research Meeting, p. 102.

Milligan III, W.H. and R.D. Fletcher. 1971. Failure to Detect Poliovirus Replication in Human Gingival Cell Cultures. Internat. Assoc. for Dental Research Meeting, p. 103.

Milligan III, W.H. and R.D. Fletcher. 1971. Rhinovirus Ribonucleic Acid Synthesis in Actinomycin D-treated KB Cells. Bacteriol. Proc., p. 172.

Current Publications:

Fletcher, R.D., C. Jayavasu, L. Zaner and D. Platt. 1972. Gingival Cell Propagation of Influenza Virus. Internat. Assoc. for Dental Research, p. 409.

Fletcher, R.D. and C. Jayavasu. 1972. Interaction of Herpes Simplex Virus and Rhinovirus in Cell Culture. Archiv fur die gesamte Virusforschung 38, 105-107.

Fletcher, R.D. and C. Jayavasu. 1972. Effect of Actinomycin D on Mycoplasma pneumoniae. J. Bact. (in preparation).

Fletcher, R.D., C. Jayavasu, S. OhYoo and J.N. Albertson, Jr. 1973. Inhibition of Mycoplasma pneumoniae by Actinomycin D. Bacterial Proc., in press.

Milligan III, W.H. and Fletcher, R.D. 1972. Rhinovirus RNA Synthesis in Actinomycin D-Treated KB Cells, (in preparation).