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

ANNUAL REPORT

By

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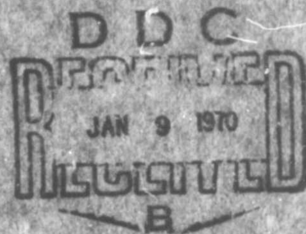
December 1969



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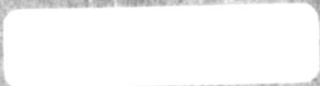

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SUMMARY

It has been demonstrated that Rhinovirus ribonucleic acid (RNA) synthesis was greater in *Mycoplasma pneumoniae* inoculated KB (Human Carcinoma of Nasopharynx) cells than in PPLO-free tissue systems. Rhinovirus RNA synthesis in these *M. pneumoniae* pre-inoculated cells was stimulated throughout the entire period of synthesis, whereas tritiated-uridine uptake by rhinovirus in non-mycoplasma treated cultures ranged from $<$0.1% to 80% of the uptake in the mycoplasma-virus inoculated cultures at 7 to 9 hrs post-infection.

In this study, *M. pneumoniae* was grown on glass to eliminate the PPLO medium. However, if PPLO medium was added to the *M. pneumoniae* inoculum, stimulation of viral-RNA synthesis was greater than in the presence of PPLO alone. Rhinovirus-RNA synthesis, measured by uridine-^{3}H uptake, in the presence of *M. pneumoniae* and *M. pneumoniae* plus medium, was enchanced 1.2 fold and 1.4 fold, respectively, above virus synthesis in untreated KB cell systems. PPLO medium alone did not appear to significantly stimulate viral RNA synthesis. However, a 5 fold increase in the concentration of the medium or its components resulted in greater viral RNA replication. The medium components, yeast extract and PPLO broth (Difco), stimulated viral RNA synthesis 4 fold and 2 fold, respectively. Our findings also demonstrated that other components, agar, phenol red, and dextrose, inhibited viral replication, whereas horse serum appeared to have no effect.

With respect to influenza virus, A/PR8, grown in Rhesus monkey kidney (RMK) monolayers pre-inoculated with *M. pneumoniae*, a two-fold stimulation of HA titers was observed when compared to virus grown in

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RMK cells without mycoplasma.

In preparation for adenovirus-mycoplasma studies, DNA synthesis of mycoplasma in human gingival and HeLa cell culture was studied. *M. salivarium* uptake of thymidine-^{3}H was approximately 4 fold greater than that of *M. pneumoniae* in both cell systems.

INTRODUCTION

The major objective of this work was to provide basic information on the interaction of mycoplasma with selected respiratory viruses. Because of our interest in rhinovirus replication, we determined the effect of mycoplasma on these infectious agents *in vitro*. In these initial studies of viral-mycoplasma relationships, we measured viral ribonucleic acid (RNA) synthesis by the rate of incorporation of uridine-^{3}H. *Mycoplasma pneumoniae* and rhinovirus 2060 were the microbial agents employed, since both are respiratory tract pathogens in man. In addition to stimulation of viral replication by *M. pneumoniae*, viral enhancement was also observed as a result of PPLO-medium components. This annual report describes not only the stimulatory effect on viral replication by the mycoplasma, but also the effect of the individual and combined components of the PPLO-growth medium.

Finally, the effect of mycoplasma on influenza viruses and preliminary studies with the adenoviruses (DNA synthesis of mycoplasma in gingival and HeLa cells) will be discussed. Emphasis is now being placed on adenovirus-mycoplasma interactions because of the importance of adenovirus infections in military recruits and because our research techniques have been sufficiently standardized for this study.

MATERIALS AND METHODS

RHINOVIRUS: Rhinovirus type 1A strain 2060 was initially purchased from the American Type Culture Collection. The stocks employed for our studies were prepared by infecting KB cell (human carcinoma of the

nasopharynx) monolayers in 32oz. prescription bottles in the presence of BME (2% calf serum) followed by incubation at 33C. After a 4+ cytopathic effect was observed, the virus was harvested by 3 cycles of freezing and thawing (-60C to 25C), followed by centrifugation at 5,000 RPM for 10 minutes at 4C. The supernatant fluids were then titered and stored at -60C. Rhinovirus stock was titered in KB cell monolayers at 33C on a roller drum (1 rev/min), and after 7 days, the $TCID_{50}$/ml was calculated by the method of Reed and Meunch. Virus stocks prepared in this manner had a $TCID_{50}$/ml of 10^6 to 10^7. In subsequent uridine-^3H uptake studies, the KB cell monolayers were infected at a multiplicity of infection (MOI) of 40. Virus stocks proved negative for mycoplasma when cultured on suitable PPLO medium.

MYCOPLASMA PNEUMONIAE: *M. pneumoniae* (Eaton agent) was initially procured from the American Type Culture Collection (ATCC #15293) and was propagated in PPLO growth medium consisting of 70% Difco PPLO broth, 20% horse serum (GIBCO) and 10% fresh yeast extract (GIBCO). In addition, the medium contained 0.5% dextrose (w/v) and 0.004% phenol red (w/v). *M. pneumoniae* stocks were prepared by the inoculation of approximately 5 x 10^7 acid forming units (AFU's) of *M. pneumoniae* into 32oz. sterile prescription bottles containing 15 ml of the above PPLO growth medium. The inoculated bottles were then placed horizontally in a 37C incubator. After a suitable period of incubation (usually 4-5 days), the supernatant fluid was decanted, and the confluent *M. pneumoniae* colonies adhering to the glass were rinsed three times with Earles salt solution containing 2% calf serum and 0.85% $NaHCO_3$. The bottles were drained and the adherent colonies resuspended in the aforementioned rinse solution by scraping the *M. pneumoniae* colonies from the glass

surface with a rubber policeman. The titer of the resultant M. pneumoniae grown on glass (MP/G) was then determined in triplicate by making serial tenfold dilutions of the MP/G in PPLO medium contained in sterile screwcap tubes (16 x 125mm). After 21 days, at 37C the acid-forming units/ml were calculated by the method of Reed and Meunch. The MP/G stock was employed throughout this study, because of the stimulatory effect of PPLO growth medium in the presence of mycoplasma on rhinovirus RNA replication reported by Fletcher, Milligan and Albertson (Bull. Czech. Soc. for Microbiol. and Folia Microbiol., in press).

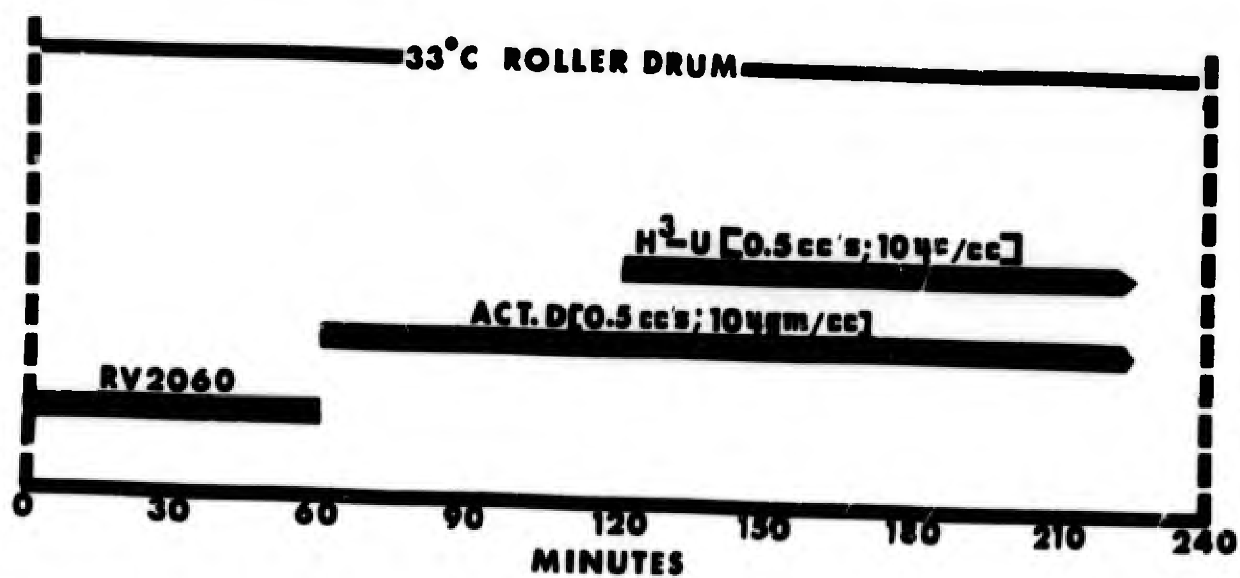
TISSUE CULTURE: The KB cell monolayers were purchased from Flow Laboratories, Rockville, Maryland in 16 x 125mm screwcap tubes. These cells were used as a source of starter cells for preparing monolayers in 32oz. prescription bottles and also for assaying viral RNA synthesis. Media for growing the cells consisted of Earles tissue culture medium (BME) with glutamine, supplemented with 10% calf serum, while media used for maintenance of the cells was BME supplemented with 2% calf serum. Neither the growth nor the maintenance media contained antibiotics. Periodically, the cells were tested for the presence of PPLO contamination by inoculation of cell suspensions on PPLO agar plates.

Assay Procedure. The effect of M. pneumoniae, PPLO medium, and M. pneumoniae plus PPLO medium, on rhinovirus 2060 nucleic acid synthesis in actinomycin D inhibited KB cells was measured by uridine-^{3}H uptake. At minus 12 hr, KB-monolayer containing tubes were drained and 0.9 ml of BME was added/tube. These tubes were divided into 5 groups: 1. inoculated with 0.1 ml of M. pneumoniae stock in BME; 2. inoculated with 0.1 ml of M. pneumoniae stock in PPLO medium; 3. inoculated with

0.1 ml of PPLO medium; and, 4 and 5 inoculated with 0.1 ml of BME. Then all tubes were incubated at 37C for 12 hr. At the end of this incubation period (zero time) the tubes were drained and group 1, 2, 3 and 4 were inoculated with 1 ml of rhinovirus 2060 and to group 5 (uninfected tissue control) was added 1 ml of BME. Incubation continued for 1 hr at 33C, and then all tubes were drained, each tube washed with 1 ml of BME, and 0.5 ml of BME containing actinomycin D (5μg/0.5 ml) was added/tube. One hundred and twenty minutes after the virus inoculation, uridine-^{3}H (specific activity: 8.0 c/mmole) was added to give a final concentration of 5μc/ml (Figure 1). Incubation continued at 33C on a 3 rev/min roller drum. Then tubes containing infected-KB monolayers were selected from each group for processing at 5, 6, 7, 8, 9, and 11 hr post-infection. Processing was accomplished as follows: triplicate tubes of each group were drained, washed 3 times with 4 ml of 5% trichloroacetic acid, drained, and the tissue sheets solubilized in 0.5 ml of hydroxide of hyamine and 10 ml of scintillation solution (2, 5 diphenyloxazole, 4 gms; 1, 4-bis-[2-(4-methyl-5-phenyloxazolyl]-benzene, 200 mg; toluene, 950 ml; and absolute ethanol, 50 ml). Activity/sample was measured in a Packard Tri-Carb scintillation counter, model 3320. Isotope activity of infected KB cell systems minus uridine-^{3}H uptake values of actinomycin D treated (uninfected) tissue controls was reported.

The stimulatory effect of PPLO growth medium and each of its components was demonstrated by adding 0.1 ml of individual components at the concentration normally in PPLO medium, and in another study, 0.1 ml of a 5 fold concentrated PPLO medium or its components. Individual PPLO-

Figure 1. Sequence of rhinovirus, actinomycin D, and uridine-^{3}H additions to KB monolayer systems



medium components, normal or 5 fold concentration, were added to appropriate volumes of BME. The effect of each component and the complete PPLO medium was assayed as described above, except for the various additions of media, which were introduced into the KB monolayer systems at zero time with the virus. Following incubation, 8 hr post-infection, these samples were processed and counted for radioisotope activity.

DNA synthesis of mycoplasma was measured in human gingival cell culture (Smulow and Glickman. 1966. Proc. Soc. Exp't. Biol. Med. 121: 1294) and HeLa cell culture. These tissue containing tubes were drained and divided into three groups, which were inoculated with 0.5 ml of *M. pneumoniae* (10^7 AFU/ml), *M. salivarium* (10^8 CFU/ml) or PPLO medium, respectively. Then 0.5 ml of thymidine-^{3}H was added to each tissue system for a final concentration of 2μc/ml. Samples were collected at selected times following the mycoplasma inoculation and the radioisotope activity was measured in a Packard Tri-Carb scintillation counter.

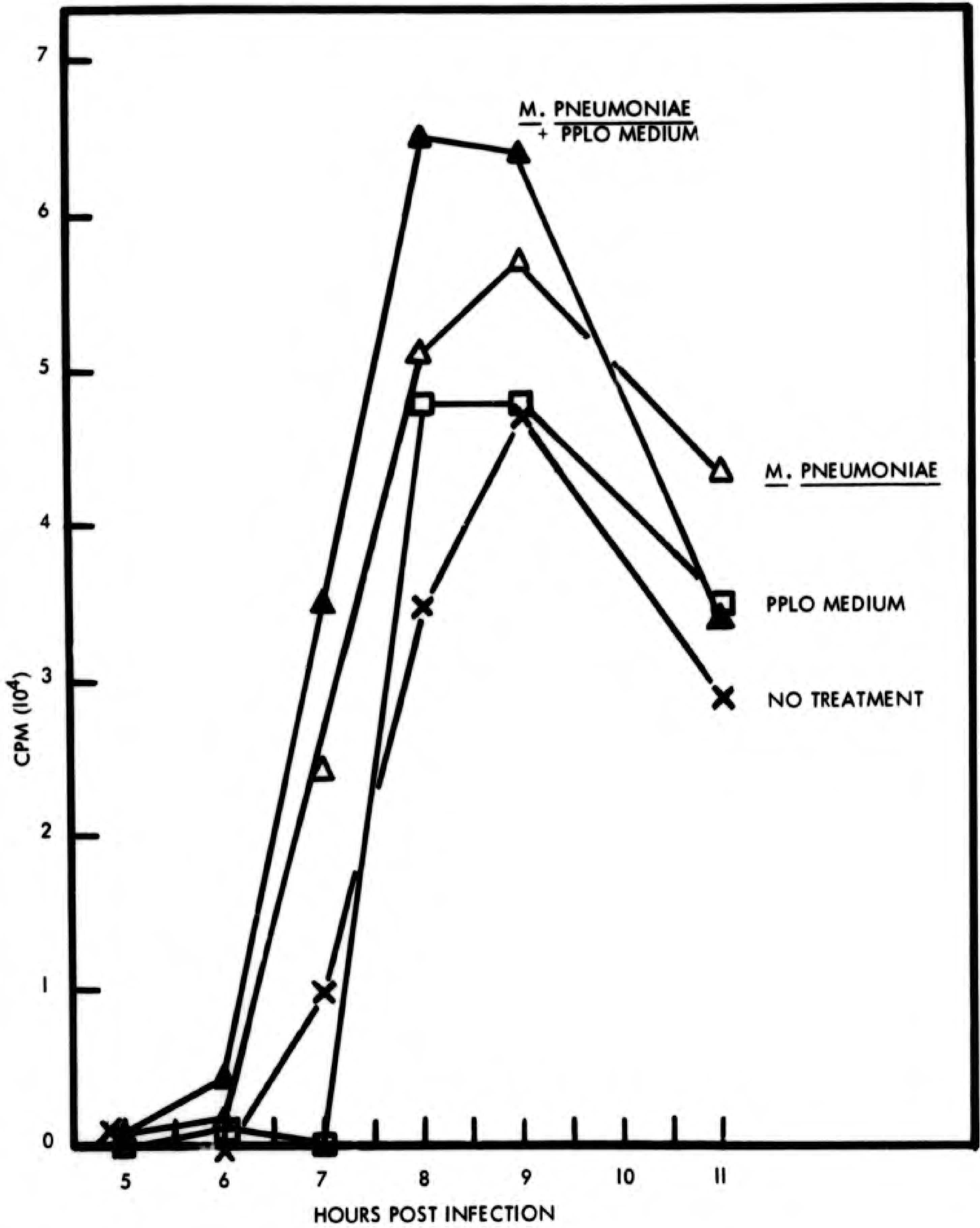
RESULTS

Rhinovirus-RNA synthesis, measured by total uridine-^{3}H uptake from 5 to 11hr post infection, was enhanced by *M. pneumoniae* plus PPLO medium and *M. pneumoniae* in Earle's maintenance medium (BME), by 1.37 and 1.20 fold, respectively, above the untreated-rhinovirus-infected KB systems (Fig. 2). These peaks of viral-RNA synthesis appeared at 9 hr post-infection, except for the *M. pneumoniae* plus PPLO medium treated cells, which showed greatest uridine-^{3}H uptake at 8 hr post-infection.

As described above, PPLO medium and *M. pneumoniae* appreciably enhanced viral RNA synthesis, but in contrast the PPLO medium alone produced no apparent stimulation. Individual components of the PPLO medium, together

Fig. 2. Enhancement of rhinovirus 2060-RNA synthesis by *Mycoplasma pneumoniae* and *M. pneumoniae* plus PPLO medium added at minus 12 hr prior to viral infection of KB monolayers. PPLO medium alone was also added to the KB monolayer systems at minus 12 hr. Following the minus 12 hr treatment all KB systems were incubated at 37°C. At zero time, the pre-treated and untreated systems were infected with 1 ml rhinovirus 2060 (MOI=40), and incubated at 33°C on a roller drum (3 rev/min) for 1 hr. Then the infected-KB monolayers were drained and washed with 1 ml of Earle's maintenance medium (no serum), drained, and treated with 0.5 ml of actinomycin D (5μg/ml). Incubation was continued at 33°C in the 3 rev/min roller drum. At two and one-half hr post-infection, 0.5 ml uridine-^3H was added to each KB infected system to give a final concentration of 5μc/ml. Incubation continued at 33°C on the roller drum 3 rev/min. Samples were collected at 5, 6, 7, 8, 9, and 11 hr post-infection, processed, and counted for radioisotope activity.

Figure 2. Enhancement of rhinovirus-RNA synthesis by M. pneumoniae and M. pneumoniae plus PPLO medium.



or individually. in BME produced little effect on viral RNA synthesis, except for the stimulatory effect of PPLO broth, and the inhibitory effect of agar and dextrose (Table 1). The PPLO medium and its individual components were concentrated 5 times before addition to the KB monolayer systems and subsequently were inoculated with rhinovirus 2060. The concentrated PPLO medium or components effected viral-RNA synthesis as follows: there was enhancement due to PPLO broth, fresh yeast extract and the complete PPLO medium by 1.74, 4.10 and 2.81 fold respectively; there was inhibition due to purified agar, which was complete, while dextrose and phenol red inhibited by 0.74 and 0.18 fold, respectively; and horse serum had no apparent effect.

The effect of various MOI's of M. pneumoniae inoculums on rhinovirus RNA synthesis in KB cells is demonstrated in table 2. Stimulation was observed at 16.5, 8.25, and 4.1 AFU's of M. pneumoniae/cell, whereas apparent inhibition was observed at 33 AFU's/cell. Thirty-three AFU's of M. pneumoniae were observed to have no effect on the number of viable KB cells throughout the 12 hr period of exposure. We have been unable to demonstrate stimulation of viral-RNA synthesis with 3 AFU's or less of M. pneumoniae at 12 hours pre-viral infection. Experiments are now in progress to define the MOI of rhinovirus and the AFU of M. pneumoniae necessary to give maximal stimulation of viral nucleic acid replication.

Preliminary experiments on the effect of different times of pre-exposure of KB cells to M. pneumoniae inoculum prior to viral infection indicate that viral nucleic acid stimulation occurs when M. pneumoniae

Table 1. Effect of PPLO medium and each PPLO medium component on uridine-^{3}H (5μc/ml) uptake by rhinovirus 2060 in actinomycin D (5μg/ml) inhibited KB monolayer systems.

TREATMENT*	COUNTS/MINUTE			
	PPLO Medium or Medium Component			
	(normal concentration)		(concentrated 5x)	
PPLO Broth (Difco)	10,250	(1.36)**	3,854	(1.74)**
Horse Serum	6,453	(0.86)	2,107	(0.95)
Fresh Yeast Extract	7,354	(0.98)	9,092	(4.10)
Agar, Purified (Difco)	5,962	(0.79)	0	(0.00)
Dextrose	5,680	(0.75)	1,638	(0.74)
Phenol Red	7,974	(1.06)	388	(0.18)
PPLO Medium (All of Above Components)	6,904	(0.92)	6,230	(2.81)
NONE	7,524	--	2,216	--

*Each treatment (0.1 ml) was made to the KB monolayer system 15 min. prior to the virus (0.9 ml) addition.

**The degree of stimulation or inhibition compared to the virus control (no treatment).

TABLE 2. Effect of Various Concentrations of *Mycoplasma pneumoniae* grown on glass on Rhinovirus 2060 RNA Synthesis in KB cells.

MOI *MYCOPLASMA PNEUMONIAE*	PEAK OF RHINOVIRUS RNA SYNTHESIS	
	TIME (HOURS)	% STIMULATION
33.0	10	0
16.5	11	7
8.25	10	10
4.1	10	9
2.1	11	0
NONE	10	-

is inoculated at 48 or 12 hours prior to viral infection, but is not evident when M. pneumoniae is inoculated at 24 hours prior to and at the time of virus infection.

It was initially thought that the M. pneumoniae inoculum might be carrying a soluble factor which was responsible for the stimulation, and to test the possibility, a portion of the M. pneumoniae stock (suspended in BME, 10% calf serum) was filtered through a 0.45mμ filter. The filtrate was cultured for viable M. pneumoniae and no mycoplasma were detected. This filtrate was then used to pretreat KB cells 12 hours prior to virus infection. No effect upon viral-RNA synthesis was observed and the pattern of viral RNA synthesis in the "filtrate-treated cells" was identical to that of the virus controls.

Initial studies with other mycoplasmas and rhinovirus in HeLa cells were as follows: M. laidlawii failed to exert any measurable effect on subsequent rhinovirus infections based on uridine-^{3}H incorporation. However, M. salivarium reduced total rhinovirus RNA synthesis in HeLa systems. The effect of these two mycoplasma on other respiratory viruses will be investigated in comparison with M. pneumoniae.

Influenza A/PR8 grown in Rhesus monkey kidney (RMK) inoculated with M. pneumoniae was stimulated two fold over influenza grown in RMK without PPLO. This enhancing effect was measured by hemagglutination titration with sheep RBC. M. pneumoniae alone in RMK produced no HA titer.

Survival of adenovirus, strain 7, at 37C in the presence of M. pneumoniae did not appear to be increased over the virus alone in maintenance medium. However, stimulation of viral DNA synthesis by PPLO is possible. This will be measured by the rate of thymidine-^{3}H uptake by these DNA viruses (adenovirus, strain 3, 4 and 7).

In preparation for continued studies with adenoviruses, DNA synthesis of mycoplasma in non-inhibited cells was measured. Thymidine-^{3}H uptake of M. salivarium alone plus gingival cells alone was only 20% of the activity measured for M. salivarium propagated on gingival cells. M. salivarium thymidine-uptake was approximately 4 fold greater than M. pneumoniae in gingival cells during the first 6 hours post-infection Mycoplasma DNA synthesis appeared to be slightly greater in HeLa cells than in the gingival monolayer systems, but this could be explained in part by the increased rate of thymidine uptake by HeLa cells.

DISCUSSION

The results reported describe a stimulatory effect of M. pneumoniae on rhinovirus 2060 RNA synthesis. Mycoplasma induced viral stimulation has previously been reported by Singer, Kirchstein, and Barile (Nature 222:1087-1089, 1969), working with vesicular stomatitis virus and M. arginini, and by Gafford, Sinclair, and Randall (Virology 37:464-472, 1969)

with *M. gallisepticum* and fowlpox virus. In contrast to these reports of stimulation, an inhibitory relationship has been demonstrated between selected arboviruses and mycoplasma (Kagan, et al, Voprosy Virusologii 5:600-604) and between Rous sarcoma virus and a mycoplasma-like factor by Ponten and Macpherson (Ann. Med. exp. Fenn. 44:260-264). Thus, it appears that the effect of mycoplasma on viral replication depends on both the virus and the mycoplasma.

The mycoplasma alone, or in association with KB cells, are clearly not eliciting a soluble stimulatory substance, indicating that intact mycoplasma are necessary for the stimulation.

The results of *in vitro* studies may be misleading when extended to *in vivo* conditions, but recently Kasza et al (Vet. Rec. March 15, p. 262-267) reported that inoculation of germ-free pigs with *M. hyopneumoniae* and a swine adenovirus (both of which are pathogenic for swine) resulted in a pneumonia of greater severity than either agent produced alone. These results, coupled with out initial observations may indicate that the interaction of *M. pneumoniae* with rhinovirus 2060 could result in a more severe upper respiratory tract infection.

The mode-of-action of *M. pneumoniae* and PPLO medium is presently being studied. It would appear that both *M. pneumoniae* and the medium effect an early stage of the viral infectious cycle.

Fresh yeast extract stimulation of rhinovirus RNA synthesis was dramatic and probably accounts for most of the enhancement at the 5 fold concentration of PPLO medium. Yeast extract has frequently been shown to enhance bacterial growth, but stimulation of viral replication is unique. Previously, Wittler, *et al.* (J. Gen. Microbiol. 14:763, 1956)

and Eaton et al. (J. Bacteriol. 84:1330, 1962) described increased growth of PPLO in tissue cultures supplemented with yeast extract. Possibly the greater enhancement of viral RNA synthesis by M. pneumoniae in the presence of PPLO medium could be explained by the ability of the organism to concentrate select ingredients of the medium.

The greatest inhibition of viral-RNA synthesis by components of PPLO medium was due to the presence of purified agar. Agar appears to adsorb the intact viral particles resulting in a lower level of infection. As for dextrose and phenol red, inhibition would be expected at the 5 fold and higher concentrations.

CONCLUSIONS

Rhinovirus type 1A strain 2060 RNA synthesis in M. pneumoniae pre-inoculated cells was stimulated throughout the entire period of synthesis in KB cell monolayer systems. This stimulation was observed at 16.5, 8.25 and 4.1 AFU's of M. pneumoniae/cell, whereas apparent inhibition was observed at 33 AFU's/cell. If PPLO medium was added to the M. pneumoniae inoculum, stimulation of viral RNA was greater than in the presence of PPLO alone. With another virus-cell system, influenza A/PR8,virus hemagglutination titers were increased when the Rhesus monkey kidney cells were pre-treated with M. pneumoniae.

In preliminary studies with adenovirus strain 7, the survival of the virus at 37C in the presence of M. pneumoniae was not increased over the survival of the virus in tissue maintenance medium.

RECOMMENDATIONS

Now that methods have been developed for investigation of virus-mycoplasma interactions based on nucleic acid synthesis, it is recommended that emphasis be placed on studies of adenovirus-mycoplasma interactions. Adenoviruses strain 3, 4 and 7 have been selected because of their importance in causing respiratory diseases, especially in military recruits. (Berge et al. 1955, Am. J. Hyg. 62:283-294; Arlander et al. 1965. Am. J. Public Health 55:67-80; Pierce and Miller, 1965. Am. J. Public Health 55:60-61; Rosenbaum, et al. 1965. Am. J. Public Health 55:38-46; Sherwood, et al. 1961. J.A.M.A. 17:1125-1127). These adenoviruses will be interacted with Mycoplasma pneumoniae, another important causative agent of respiratory disease in military recruits (Chanock, Fox and James. 1967. Ann. N.Y. Acad. Sciences 143:484-496).

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REPORTS AND PUBLICATIONS

Milligan III, W.H. and R.D. Fletcher. 1970. The effect of Mycoplasma pneumoniae on rhinovirus-RNA synthesis in KB cells. Antimicrobial Agents and Chemotherapy--1969, in press.

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 Microbiol. 5:34.

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Fletcher, R.D. and R.A. Johnson. 1970. DNA synthesis of mycoplasma in human gingival cell culture. International Association for Dental Research, submitted for publication.

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13. ABSTRACT

Studies were performed to determine the effect of Mycoplasma pneumoniae on rhinovirus replication in KB cells. Rhinovirus-RNA synthesis in KB cell monolayers was measured by tritiated-uridine uptake in the presence of actinomycin D. It was observed that viral-RNA synthesis in M. pneumoniae pre-inoculated cells was stimulated throughout the entire period of synthesis, whereas triated-uridine uptake by rhinovirus in non-mycoplasma treated cultures ranged from $< 0.1\%$ to 80% of the uptake in the mycoplasma-virus inoculated cultures at 7 to 9 hours post-infection. In these studies, M. pneumoniae was grown on glass to eliminate the PPLO medium. However, if PPLO medium was added to the mycoplasma inoculum, stimulation of viral RNA synthesis was greater than in the presence of mycoplasma alone.

Influenza virus, A/PR8, grown in Rhesus monkey kidney monolayers inoculated with M. pneumoniae, showed a two fold stimulation of HA titers greater than virus grown in RMK cells without mycoplasma.

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