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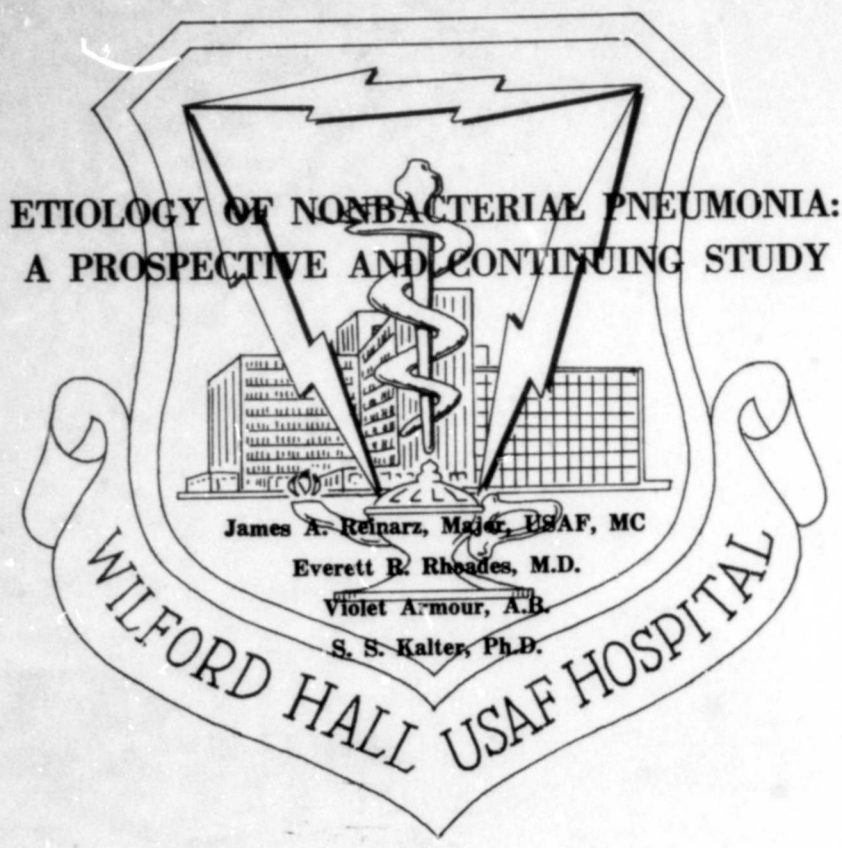

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FOREWORD

This report was prepared in the Infectious Disease Section, Department of Medicine, Wilford Hall USAF Hospital, and in the Division of Microbiology in Infectious Diseases, Southwest Foundation for Research and Education, San Antonio, Texas, under Research Work Unit 775602006 monitored by the Aerospace Medical Laboratory (Clinical). It was submitted for publication in December 1967. The work was accomplished between January 1964 and December 1966.

This report has been reviewed and is approved.

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ABSTRACT

Nonbacterial pneumonia occurs frequently in military populations, especially among recruits. The morbidity is high in otherwise healthy individuals and the time lost from training or duty is significant, particularly to the recruit, but deaths are rare. The known causes of nonbacterial pneumonia vary by season and geographical location from year to year. In January 1964 a three-year study was undertaken to determine the prevailing causes in patients admitted to Wilford Hall USAF Hospital. Acute sera, a throat wash and a sputum specimen were obtained from each patient as soon as possible after admission and prior to antimicrobial therapy. Convalescent sera were collected approximately two weeks later and again whenever possible. Of 1,053 patients studied, only 740 were available for detailed analysis. Adenoviral pneumonia, the specific entity most frequently diagnosed (14.7 per cent), was confined largely to basic trainees. Mycoplasma pneumoniae, confirmed etiologically in only 9.9 per cent, was associated invariably with a diagnostic serologic response. Other specific agents, including influenza (types A and B), Coxiella burnetii (Q fever), respiratory syncytial virus and various parainfluenza strains, constituted 9.3 per cent. Nondiagnosed, nonbacterial pneumonias composed the largest single group (66 per cent), possibly because of the inadequacy of current laboratory techniques or the presence of agents not identified previously.

LIST OF ABBREVIATIONS

PPLO	Pleuropneumonia-like organisms
C	Centigrade
TCD	Tissue culture dose
ECHO virus	Enteric cytopathogenic human orphan virus

PROBLEM

Nonbacterial pneumonias are responsible for significant morbidity in otherwise healthy individuals (1-4). In rare instances death may result (5, 6). Nonbacterial pneumonia may occur in any population, but it is particularly frequent in military populations and even more so in military recruit populations (1, 7-9). The morbidity in these groups is significantly high. At Lackland Air Force Base, Texas, the largest basic military training center in the United States Air Force, prior experience has indicated that 200 cases of nonbacterial pneumonia per year may be expected. If one assumes a duration of illness of 14 days, 2,800 man-days are lost per year. Furthermore, loss of time from duty or training, deconditioning and muscle atrophy as a result of illness and bed rest, and post-infection asthenia are even more significant causes of relative ineffectiveness.

Although nonbacterial pneumonia is recognized as a problem in basic military trainees, it is at least as significant in other active duty personnel and their dependents. They also experience loss of time from duty and ineffectiveness.

Known causes of nonbacterial pneumonia include Mycoplasma pneumoniae (PPLO, Eaton agent), several adenoviral strains (usually types 4 and 7), influenza (types A and B), Coxiella burnetii (Q fever), possibly parainfluenza (types 1, 2 and 3), and respiratory syncytial virus (3). In several series, these agents have been confirmed etiologically in 50 to 70 per cent of nonbacterial pneumonias. However, a large percentage remains undiagnosed in any series.

The absolute numbers and the relative incidence of these agents vary from year to year by geographic location and by season (10-12). These are recognized variables, but it is not possible to extrapolate the etiologic agents and their frequency for Lackland Air Force Base or any other location from published data. In order to understand the problem at this installation and in this geographic area, it was necessary to determine the prevailing causes of pneumonia in patients admitted to Wilford Hall USAF Hospital. This study was begun in January 1964. In this

manner, and only in this manner, can the causative agents be identified, their relative frequency be determined, and the magnitude of the problem be assessed. Without this information no intelligent control measures can be implemented. Rational therapy can be instituted only when it is directed against reasonably likely agents.

Effective antimicrobial treatment is available for Mycoplasma pneumoniae (9, 11, 13) and Coxiella burnetii; however no chemotherapy is effective for adenoviruses, influenza, parainfluenza, or respiratory syncytial virus. Antimicrobial chemotherapy is not only ineffective but it may be deleterious for these infections. Antiviral chemotherapy is a possibility (14). Effective chemoprophylactic agents are available for influenza A2 now (15, 16), and if it were to represent an appreciable problem, chemoprophylaxis might be possible. Although it is the only respiratory viral agent for which prophylactic therapy exists, others should be forthcoming.

Immunization to the various respiratory agents, including Mycoplasma pneumoniae, multiple types of adenovirus, influenza, parainfluenza, and respiratory syncytial virus, is undergoing clinical trial (17-23). Preliminary data indicate that the vaccines, both attentuated live and killed, are effective in producing serologic response and in protecting the individual from subsequent infection. However, immunization can be directed only against known agents, preferably those known to cause disease in that community.

Unfortunately, available technology allows only for retrospective diagnosis of nonbacterial pneumonia. It is not sufficient to demonstrate or isolate an agent in order to relate it causally with an infection. Proof of the infection is possible only by demonstration of a serologic response to that agent in the host. Preferably, the serologic response (a fourfold or greater increase) to an isolated agent should be demonstrated. This is an idealized scheme, but isolation of an agent from the respiratory passage, throat, or stool without a demonstrable rise in antibody titer indicates only the presence of the organism and cannot be equated with infection.

Therefore, the primary means of diagnosis for the present patients was serologic. The major emphasis was on previously recognized agents. Therefore, viral and mycoplasmal isolation was to be confirmatory, and the identification of previously unrecognized pathogens secondary.

MATERIALS AND METHODS

Patients

Description of the patients and the clinical findings will be reported separately. The majority of the patients were airmen in basic training; however, other Air Force personnel and their dependents were included, primarily for comparative purposes. Over the three-year period 1,053 patients were studied.

Collection of Specimens

Acute sera and specimens from both bacteriology and virology were obtained as soon as possible after admission to the hospital and prior to antimicrobial therapy. The specimens from adults consisted of a throat wash and a sputum specimen collected in the morning and prepared for study the same day, i.e., ususally within three or four hours. Convalescent sera were collected approximately three weeks thereafter. All sera were maintained at -20 degrees C until tested.

Virus Isolation

Throat washes, throat swabs and rectal swabs were handled as described elsewhere (24). When suitable cells were available, the supernatant fluid was stored at -20 degrees C. Centrifugation sediments from throat washings or swabs and sputum specimens were used for bacteriological studies and placed in PPLO broth.

The virology specimens, i.e., throat washes, throat swabs and rectal swabs, were passaged on primary (usually African

green) monkey kidney cells and at least two secondary human cell lines, i.e., HeLa, Hep-2, KB, and WI-38 (human diploid cells). Specimens on a particular cell line were considered negative after three passages without development of any characteristic cytopathic effect. Isolates recovered from cell passages were titrated to determine infectivity. Titrations were performed in two tubes per dilution to determine 100 TCD_{50} for neutralization and identification studies. All isolates were subjected to neutralization tests against the patient's acute and convalescent sera.

Inasmuch as several of the isolates, especially in monkey kidney cells, were poliovirus type 1 and herpes-type viruses, all isolates were screened against a standard diultion of antisera for these two viruses. Isolates not eliminated with these antisera were exposed to pools of enterovirus antisera. These antisera pools were prepared according to methods described by Lim and Benyesh-Melnick (25), and included 1:20 dilutions of the following National Institute of Health reference antisera: poliovirus types 2 and 3; Coxsackie virus A9, Coxsackie viruses B1-B6, and ECHO virus types 1-9, 11-15, 17-20, 22-27, and 29-32. After inhibition of the cytopathic effect of pooled sera, the isolate was identified by specific neutralization tests.

Serum pairs (acute and convalescent) were tested for complement fixation against a battery of viral antigens previously known to cause respiratory disease, including adenovirus (group antigen), parainfluenza (types 1, 2, and 3), respiratory syncytial virus, influenza (types A and B), Coxiella burnetii (Q fever), and Mycoplasma pneumoniae (PPLO, Eaton agent). The complement fixation test was performed according to the procedure previously described (5). It is to be emphasized that a fourfold or greater increase in antibody titer was demanded for seropositive diagnosis. No empiric dilution or antibody titer fall was considered seropositive.

Bacteriologic specimens included two separate aerobic blood cultures, a sputum culture and, occasionally, a culture of transtracheal aspirate and/or pleural fluid. Blood culture bottle (trypticase soy broth) was held ten days and then subcultured to solid media. Sputum cultures were plated on 5 per cent sheep blood and eosin methylene blue agar, observed for growth at 24 hours, and then discarded. No anaerobic cultures were performed.

Mycoplasma agar and broth were prepared and used according to the procedures recommended by Hayflick and Chanock (26), employing PPLO agar and broth, agamma horse serum, a fresh extract of yeast, and a 1:50 dilution of thallium acetate and penicillin. These were incorporated into the media for the initial plating of the specimen to inhibit growth of all other microorganism

When Mycoplasma species were isolated, one or more clones were subcultured to new agar plates. Inhibition discs, saturated with approximately 0.25 ml of undiluted antisera to the various species of human Mycoplasma, were added to the plates for identification of the species. Sheep cell overlay was added to positive plates at the end of 48 hours of good growth to determine hemolysis (27).

RESULTS

Viral Isolation

Four isolates were recovered from the 1964 respiratory specimens on Hep-2 cells (Table I). Two of these were identified as adenovirus type 4. A significant rise in neutralizing antibody against adenovirus type 4 was observed when the acute and convalescent sera from one patient and the convalescent serum from another were tested against this virus. The other two isolates were shown to be poliovirus type 1 by neutralization tests.

Thirteen isolates were obtained in 1965 on monkey kidney cells and consisted of one ECHO virus type 6, three polioviruses type 1, and nine Herpes-type viruses. Five of these isolates were recovered on WI-38 cells also. Unfortunately, the ECHO virus type 6 isolate could not be associated with the clinical disease, inasmuch as no sera were obtained on the patient. It is of interest to note that two separate throat washings on one patient, obtained approximately one month apart, yielded the same organism (Herpes simplex).

TABLE I

Specific viral isolates

Year	Isolates	Positive serology
1964	*2 Adenovirus, type 4	2+
	2 Poliovirus, type 1	--
1965	1 ECHO, type 6	--
	3 Poliovirus, type 1	--
	9 Herpes simplex	--
1966	*4 Adenovirus, type 7	4+
	4 Poliovirus, type 1	--
	2 Poliovirus, types 1 and 3	--
	3 Herpes simplex	--
	1 ECHO, type 9	--
	1 Coxsackie B-3	--

*Responsible for nonbacterial pneumonia.

The 1966 isolations generally resembled those of the previous year, except that four adenoviruses were isolated. However, in contrast to previous years, all were adenovirus type 7. The four adenoviruses were recovered on HeLa cells and/or primary human amnion cells. Four viruses have been identified as poliovirus type 1 and three viruses as Herpes simplex. Other virus isolates included one ECHO virus type 9, Coxsackie virus B-3, and two specimens of poliovirus types 1 and 3.

All adenovirus isolates were tested for hemagglutination against type O human cells, rat cells and rhesus monkey cells. In addition, these isolates were subjected to complement fixation tests to demonstrate the presence of the adenovirus group antigen. In all instances, these viruses conformed to the characteristics attributed to the adenoviruses.

Serology

The acute and convalescent sera for 740 patients were assayed for antibodies to adenoviruses, Mycoplasma pneumoniae, influenza (types A and B), parainfluenza (types 1, 2, and 3), Coxiella burnetii (Q fever), and respiratory syncytial virus. Many of these sera have been tested against the Bedsonia group (psittacosis) also. Because they have been consistently negative, the results are not tabulated.

Incidence

The incidence of nonbacterial pneumonia by month is shown for a three-year period in Figure 1. Of 1,053 cases of nonbacterial pneumonia studied initially, 740 cases were available for detailed analysis. The monthly incidence of pneumonia was variable, with a peak of 35 cases in March 1964 and 34 cases in March 1965. The mean incidence was 21.8 cases per month (range, 3-35), excluding January and February in 1964. This time interval was excluded because technical problems in data collection were encountered at the inception of the study.

Etiology of Pneumonia

Analysis of these data indicated that the adenoviral pneumonia was the single most frequently diagnosed specific entity (14.7 per cent) (Table II). The overwhelming majority of cases occurred from January through April in 1964, 1965 and 1966. Only sporadic cases of adenoviral pneumonia were diagnosed during the other months.

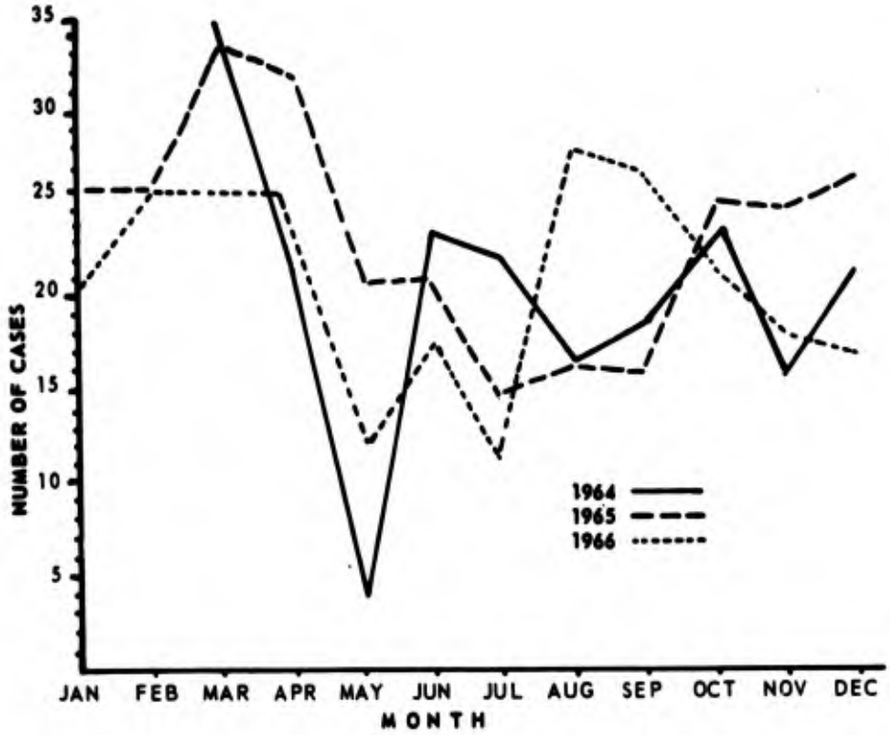

FIGURE 1

Monthly incidence of nonbacterial pneumonia, 1964-1966.

Mycoplasma was confirmed etiologically in only 9.9 per cent of the patients. This was a significant entity although the incidence was less than anticipated from previous studies (4, 7-9, 28). The seasonal distribution of pneumonia due to Mycoplasma appeared to be totally at random, with its incidence relatively constant throughout the calendar year. The incidence increased from 3.0 per cent in 1964 to 10.2 per cent in 1965, and 15.1 per cent in 1966.

Other specific agents incriminated were influenza (types A and B), Coxiella burnetii (Q fever), respiratory syncytial virus, and the various parainfluenza strains. They were randomly distributed, and no single one constituted a significant cause of nonbacterial pneumonia. Overall, this composite group was

TABLE II

Annual incidence of nonbacterial pneumonia

	1964		1965		1966		Total	
	No.	%	No.	%	No.	%	No.	%
Nondiagnosed	146	71.9	187	65.6	157	62.3	490	66.2
Mycoplasma	6	3.0	29	10.2	33	15.1	73	9.9
Adenovirus	38	18.7	44	15.4	26	10.3	108	14.6
Other specific	13	6.4	25*	8.8	31+	12.3	69	9.3
TOTAL	203		285		252		740	

*Predominately influenza A

+Predominately influenza B

responsible for 9.3 per cent. In 1965 influenza type A was predominant and in 1966 influenza type B, corresponding to their general prevalence in the United States at that time.

The largest single group consisted of the nondiagnosed, nonbacterial pneumonias comprising 66 per cent of the overall cases. The incidence was relatively constant throughout the study period, with 146 cases in 1964 (71.9 per cent), 187 cases in 1965 (65.6 per cent), and 157 cases in 1966 (62.3 per cent). The average number of cases was 14.4 per month (range, 3-23).

DISCUSSION

It is apparent that viral isolation was of little value in the diagnosis of the pneumonias in this patient group. In 1964 only

four viral isolates were obtained and only two of these, both adenovirus type 4, could be implicated etiologically in pneumonia. During this same interval, 38 patients had serologically confirmed adenoviral infection. The isolation yields were low (5.3 per cent). The other two viruses isolated in 1964 were poliovirus type 1, whose role must remain speculative.

In 1965, 13 viruses were isolated. Nine were Herpes simplex, three poliovirus type 1, and one ECHO type 6, none of which have been shown to produce nonbacterial pneumonia. No specific viral isolates were obtained corresponding to the nonbacterial pneumonia during the same interval. However, 44 cases of adenoviral, 15 cases of influenza type A, and 4 cases of influenza type B pneumonias were diagnosed serologically.

In 1966, 15 viruses were isolated. However, only the four adenoviruses have been associated with nonbacterial pneumonia previously. Confirmatory isolation was 15.4 per cent for this entity because 26 adenoviral infections were diagnosed serologically. Six isolates were the various polioviruses, one ECHO type 9, and one Coxsackie virus B-3. Three isolates were Herpes simplex.

The isolation of viral agents was of inconsequential value in this study. Only six isolates, all adenovirus types 4 and 7, could be incriminated etiologically. No new viral agents and no other known viral agents were isolated as causes of primary atypical pneumonia.

The role of the poliovirus was speculative, for no attempt was made to separate the Sabin vaccine strains of poliovirus from the wild virus. The majority of this population had recently received oral poliovirus vaccine. Several individuals demonstrated serologic conversion to this agent. The isolation of an agent and the demonstration of specific antibody rise to that agent were indicative of infection. However, this was not meant to imply that the poliovirus causes pneumonia. Poliomyelitis was not diagnosed clinically in any patients during this study, and no paralytic episodes occurred in the hospitalized patients. This finding is interpreted as what is expected normally, i.e., iatrogenic infection with the attenuated strain of poliovirus, nonapparent infection

to a non-attenuated strain, or as the isolation of a cohabiter and nonspecific phase reaction to the infection.

Herpes simplex was isolated frequently. It is a common cohabiter or latent virus in man, and frequently it is activated by concurrent febrile illness. A "fever blister" might result with or without the liberation of virus and no clinical manifestations. The serologic response evidenced in certain patients might be interpreted as either an amnestic response or as a nonspecific phase reaction to a febrile illness.

Attempts to isolate viral agents as an aid to diagnosis were disappointingly unproductive. Other studies reported much more frequent viral isolation (7, 9). The reason for the difference was not apparent. All reasonable care was taken in processing specimens and in performing virologic techniques by recognized acceptable standards.

Mycoplasma isolation, which was started in 1965, was more productive than isolation of viral agents (Figure 2). Fifteen strains of Mycoplasma pneumoniae were isolated. During the same interval, 17 strains of Mycoplasma orale type 1 were isolated and one strain of Mycoplasma hominis type I. Serologic titers were not obtained on one patient from whom the organism was cultured. Of the 13 patients from whom Mycoplasma pneumoniae was isolated, 12 were serologically positive. Sixty-five patients were serologically confirmed to have Mycoplasma infections. Since isolation of the organism usually was associated with a diagnostic serologic response, isolation of Mycoplasma did not afford any appreciable diagnostic advantage.

Adenoviral pneumonia occurred in the winter and early spring and constituted the largest single entity. It was prevalent in January through April and only sporadically at other times of the year; it constituted a significant problem at this time only. This finding was particularly significant, for comparable incidence and seasonal variation occurred in the southwest, the eastern seaboard, and the most northern states. This was expected from preliminary studies and it was consistent with outbreaks of adenoviral pneumonia reported in other studies (3, 7, 9, 12). Adenoviral infection was confined largely to basic airmen, and was not

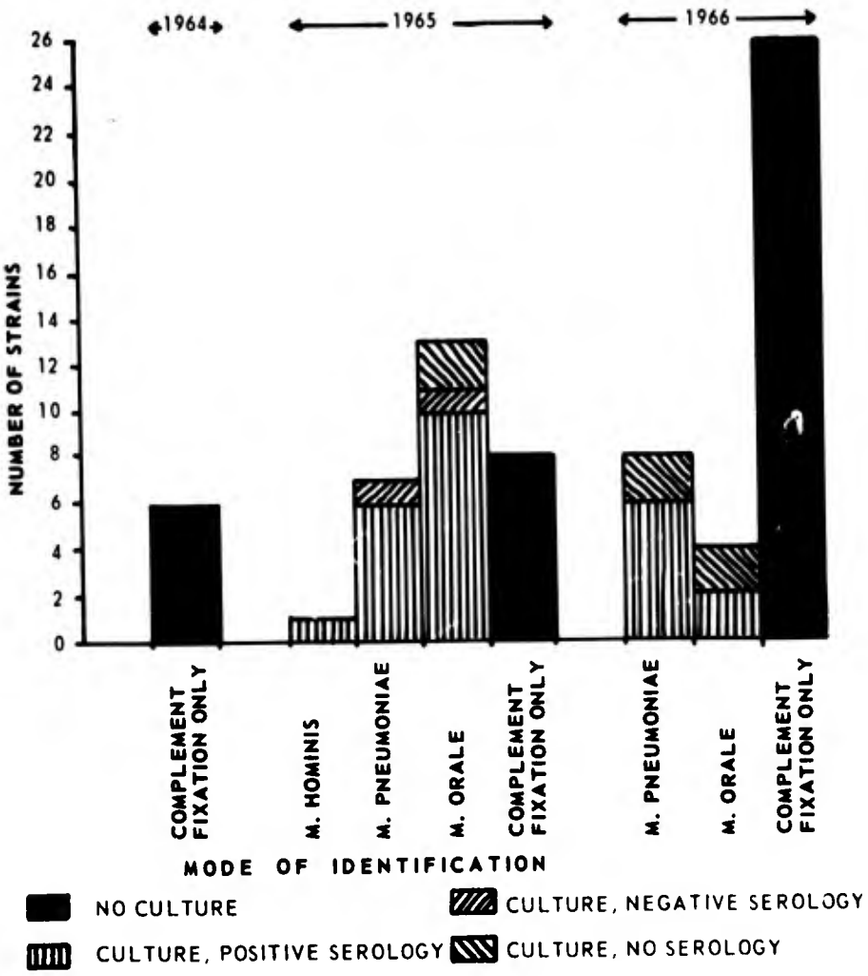

FIGURE 2

Confirmed diagnosis of Mycoplasma.

widespread in the civilian population. However, several patients were military dependents and were not basic trainees.

Mycoplasma infection constituted a lesser problem than anticipated, representing only 3.0 per cent in 1964, 10.2 per cent in 1965, and 15.1 per cent in 1966. This was explained perhaps

by the increasingly sophisticated technology or it might represent a true increase. However, even 15.1 per cent was approximately one-half of the incidence anticipated from other studies (8,9,11,28). Perhaps, insistence upon a demonstrable fourfold antibody rise lessened this percentage.

The lower incidence might be explained by an alternate thesis. All patients were admitted to the Infectious Disease Ward promptly after becoming ill. Since June 1965, a comprehensive therapeutic study comparing tetracycline and lincomycin was performed. After specimens for virus and Mycoplasma isolation and initial serum were obtained, all patients were treated with either of the antimicrobial agents in a randomized double-blind manner for ten days. It is conceivable that early appropriate therapy by mycoplasmastatic or mycoplasmacidal agents might abort the serologic response to infection significantly. This hypothesis is somewhat negated, for serologic responses occurred in 12 of the 13 patients in whom Mycoplasma pneumoniae was isolated. Both lincomycin and tetracycline have been demonstrated to be mycoplasmacidal agents (29), and it might be anticipated that they could eradicate or suppress the organisms. However, other studies have demonstrated the persistence of Mycoplasma in the sputum and throat washings in patients after chemotherapy (30). Both tetracycline and lincomycin are inhibitors of protein synthesis (31); this inhibition results in a bacteriostatic or bacteriocidal effect in microorganisms. Antibody response and serologic diagnosis are dependent upon protein synthesis. The effect of tetracycline or lincomycin has not been studied with respect to inhibition, delay or blunting other serologic responses in man, but this has been demonstrated for chloramphenicol (32). This is a speculative hypothesis, but it cannot be dismissed on the basis of available data. This mechanism has been shown to be operative in the suppression or the prevention of serologic response to group A streptococci and in patients immunized to tetanus (33, 34).

The causes of pneumonia were comparable to those in other studies. The only difference was the occurrence of a larger percentage of nondiagnosed, nonbacterial pneumonias. An alternative explanation was that this finding might represent the true incidence of these known agents at Lackland Air Force Base. This group of pneumonias might not be diagnosable with current

techniques in any laboratory at present. In fact, the monthly mean incidence of 14.4 cases (range, 3-23) remained relatively constant. There were percentage changes, not because of an absolute increase or decrease in the number of cases of nondiagnosed pneumonia, but because of the superimposition of known causes during the same period. The explanation that seemed readily apparent was that the large number of cases resulted from the inability to identify known etiologic agents because of technical inadequacies in the diagnostic laboratory. Further consideration indicated that this entity was relatively constant and might not be caused by agents known to cause nonbacterial pneumonia. Hence, there was inferential evidence that another agent (or agents) remained to be discovered. If this hypothesis is correct, Lackland Air Force Base would be a logical place to undertake a more detailed study in an attempt to identify heretofore unrecognized agents.

Within the past several years, attenuated and inactivated vaccines for adenovirus and Mycoplasma were developed (17-23); they are still undergoing clinical trials. Successful vaccines might be expected to protect susceptibles and decrease morbidity from adenoviral and mycoplasmal infection. If these data for the years 1964 through 1966 were accurate and projectable into the future, it appeared that little might be gained from a widespread vaccination program against adenovirus and Mycoplasma in this particular patient population. Perhaps, individuals undergoing basic military training between January and April should be immunized against adenoviruses. However, totally effective immunization against Mycoplasma would not affect appreciably the total number of patients with nonbacterial pneumonia at Lackland Air Force Base. A decrease of 73 in the 740 cases of pneumonia would not change the morbidity of the condition appreciably.

Therefore, the investigators urge that a more imaginative, comprehensive program be undertaken in order to identify other unrecognized agents in the undiagnosed pneumonia. This attempt employed only the simplest, most direct techniques of viral and Mycoplasma isolations. No reported attempts have been made to isolate unknown viral agents by use of viral interference techniques or by use of newer derived tissue culture cell lines. If the present large group of patients was the result of unknown viruses or

Mycoplasma, rickettsia, and other infectious agents heretofore undescribed, a wealth of information could be obtained by further studies at Lackland Air Force Base.

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13. ABSTRACT

Nonbacterial pneumonia occurs frequently in military populations, especially among recruits. The morbidity is high in otherwise healthy individuals and the time lost from training or duty is significant, particularly to the recruit, but deaths are rare. The known causes of nonbacterial pneumonia vary by season and geographical location from year to year. In January 1964 a three-year study was undertaken to determine the prevailing causes in patients admitted to Wilford Hall USAF Hospital. Acute sera, a throat wash and a sputum specimen were obtained from each patient as soon as possible after admission and prior to antimicrobial therapy. Convalescent sera were collected approximately two weeks later and again whenever possible. Of 1,053 patients studied, only 740 were available for detailed analysis. Adenoviral pneumonia, the specific entity most frequently diagnosed (14.7 per cent), was confined largely to basic trainees. Mycoplasma pneumoniae, confirmed etiologically in only 9.9 per cent, was associated invariably with a diagnostic serologic response. Other specific agents, including influenza (types A and B), Coxiella burnetii (Q fever), respiratory syncytial virus and various parainfluenza strains, constituted 9.3 per cent. Nondiagnosed, nonbacterial pneumonia composed the largest single group (66 per cent), possibly because of the inadequacy of current laboratory techniques or the presence of agents not identified previously.

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