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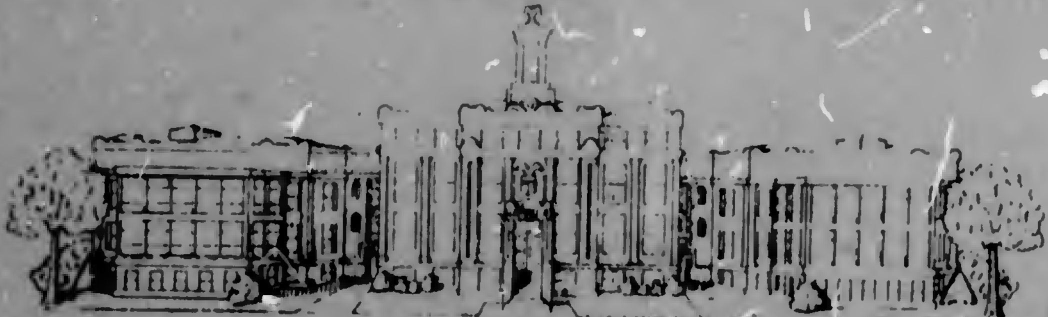

1968 RESEARCH CLERKSHIP REPORTS

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PREFACE

This report consists of scientific papers which are the consummations of research work done by the research clerks assigned to the NAMRU-4 during the summer of 1968. These research clerks were Ensigns in the Naval Reserve between their first and second year of medical school when they reported for 60 days of active duty. Due to time limitation, preliminary plans and scientific designs of the studies were done by the preceptors. Assignment of the clerk to the specific study was done to coincide as closely as possible with the interest of the clerk.

It is the philosophy of NAMRU-4 that the clerk experience all phases of the research project including: (1) library study, (2) protocol preparation, (3) collection of specimens, (4) laboratory procedures, (5) collection of data, (6) analysis of data, and (7) preparation of the final report. Close supervision and direction were given the clerks during the initial phase to insure their knowledge of the problem, objectives and techniques needed to accomplish the study. The enclosed report reflects the clerks' individual efforts. Each paper was reviewed by the respective preceptors, but changes were kept to a minimum.

To more fully indoctrinate the clerks, other opportunities were offered. The individual studies were complemented by a formal lecture series given by members of the Scientific Department to provide to all clerks the underlying concepts of microbiology, immunology, biochemistry, biometrics and allied fields. These lectures filled gaps that may have been present in the individual studies. The research clerkship is designed to be a valuable and broadening experience for the participating clerk who uses the techniques and skills needed to accomplish applied research. It is hoped that he has gained some reward along with the frustrations.

R. C. KNOWLES
LCDR MC USNR
Editor
1968 Research Clerkship Reports

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FOREWORD

It is the intent of this publication to familiarize those responsible for medical education with the results of the research clerk's (Ensign 1915 Program) experienced at Naval Medical Research Unit No. 4 (NAMRU-4). The exposure of the clerks to research methods in arriving at the reports herein contained is believed to give them a greater appreciation of investigative work and its importance to the medical sciences. The environment in which the clerks work is best portrayed by a description of the research unit.

The mission of NAMRU-4 is to conduct research on the epidemiology, etiology, and methods of prevention and control of acute communicable diseases of the respiratory tract.

NAMRU-4 offers a unique opportunity to the research clerks in offering field trials, laboratory study, and clinical evaluation, singularly or in combination, through the fine cooperation of other adjacent Naval activities including the Recruit Training Command, Service School Command, and the Naval Hospital.

The permanent staff at the research unit consists of approximately 80 individuals who are about equally divided between civilian and military ranks. The scientists are widely recognized as experts in their fields.

The consultant staff and affiliations represent such organizations as the World Health Organization, National Institutes of Health, Universities of Chicago, Illinois, Michigan, Northwestern, Tennessee, South Dakota and Wisconsin, and many of the Commissions of the Armed Forces Epidemiological Board, including those on influenza, acute respiratory diseases, streptococcus and immunization.

The unit offers its resources to the clerks in the process of conducting their investigations. Their projects are frequently not a means to an end in themselves, but are pilot studies for much larger programs. The clerks not only have the satisfaction of completing their own projects, but also may have the opportunity of realizing that they played a small part in a much larger scheme while being introduced to the philosophy and principles of the research methods.

R. O. PECKINPAUGH
CAPT MC USN
Commanding Officer

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THE ENHANCEMENT OF MOUSE VIRULENCE AND IMMUNOLOGIC CROSS-PROTECTION OF NEISSERIA MENINGITIDIS*

Ensign Joh Aldridge
Meharry Medical College

Preceptor: LCDR L.F. Devine
Chief, Bacteriology Division

Neisseria meningitidis has interested many workers for a number of years because of periodic recurrence of epidemics of meningococcal meningitis. This disease was epidemic during World Wars I and II in the United States among military populations. It remains a menace to military recruit population, (1). Naval Medical Research Unit No. 4 (NAMRU#4) is devoting attention to the causative organism with the long-range possibility of developing a vaccine which may protect against a wide range of meningococcal strains.

N. meningitidis is known to consist of several serological groups and strains. Groups A, B, C and D were designated as such in 1950 by the subcommittee on Neisseria of the Committee on Bacteriological Nomenclature of the International Association of Microbiologists (2). Since then, Slaterus (3,4) has described strains which are not agglutinated by antisera to the four major groups. These strains can be isolated from the nasopharynx of carriers and from clinical cases, and are tentatively identified as X, Y, Z and Z'. All are serologically distinct strains except Z'. The latter is not agglutinated by antiserum to Z cells, but anti-Z' antiserum will agglutinate strain Z cells. All 4 strains described by Slaterus ferment glucose and maltose, but fail to ferment levulose and lactose. They are, therefore, indistinguishable biochemically from the earlier identified serological groups. Strains of meningococci, apparently identical serologically and biochemically to those described by Slaterus, have been found repeatedly at NAMRU#4 among nasopharyngeal isolates from carrier surveys.

Evans et al (1967)(5) described another strain, 29E, isolated from a case of meningococcal disease, which appears to be serologically

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identical to the Z' described by Slaterus (6). This strain (29E) has been routinely isolated at NAMRU#4. Group E of Vedros (7), Y of Slaterus and Bo of Singer (8) appear to be identical, and are the most prevalent groups of meningococci in Naval recruit camps (9).

The serological groups and strains referred to in this study are listed in Table 1 under the NAMRU#4 column. The serological relationship of NAMRU#4 isolates to other strains or groups described in Bergey's manual (2) and recent publications are shown. Strain 17 is synonymous with G and Z, and strain 112 is synonymous with Z' and 29E. RAS 1C has no known counterpart described in literature. The sources of the strains used are noted at the foot of Table 1.

Other strains of meningococci not well described in the literature and poorly understood are meningococci agglutinated by more than one meningococcal antiserum. These strains appear to contain antigens common to several groups of meningococci, but antiserum to these strains will not agglutinate the previously described serological groups or strains. All other serological groups described in the literature have been reported as isolated from clinical meningococcal disease. However, only 1 case has been reported for group Z (10).

Greenberg et al (11) described cross-protection between actively immunized mice when challenged with heterologous serological groups of meningococci. At NAMRU#4, mice vaccinated with Bo strain antigen showed some protection against challenge by group C organisms (9). The presence of reciprocal cross-protection between serological groups or strains can be studied only with meningococci of relatively high pathogenicity for mice.

Cross-protection between strains of meningococci other than groups A, B, C and D have not been studied extensively with either active or passive immunization. Consequently, an attempt was made to increase the virulence for mice of the more recently described meningococci to test possible cross-protection by challenge of actively or passively immunized mice with heterologous strains. A meningococcal strain or group may be uncovered, which is protective against heterologous groups or strains, and be a good source of antigen for the development of a protective vaccine.

MATERIALS AND METHODS

Rapid Serial Passage of Organisms in Mice: Strains of *N. meningitidis* were stored in sheep blood at -60°. The blood samples were thawed and Mueller-Hinton agar plates were inoculated and

incubated for 18 hours. At the end of this period the organisms were taken from the plates and placed in Mueller-Hinton broth and grown for 6 hours on an incubator-shaker.

Ten-fold dilutions were made of the organisms in mucin, as described by Miller et al (12). The mucin was adjusted to a pH of 7.2 to 7.4. Selected dilutions were used to challenge mice to determine the virulence of the organisms and plate counts were made to determine the number of organisms in the inocula. The mice were challenged intraperitoneally. The number of mice that died was recorded at 24 and 48 hours. Twenty-four to 36 hours after the mice were challenged with the organisms suspended in mucin, they were bled from the periorbital venous sinus and the blood placed on Mueller-Hinton plates. The growth from this plate was then used for the next passage in a new group of mice. Each in vivo passage followed the previous in vivo passage by 48 hours. The in vitro passages were grown on Mueller-Hinton agar plates for 16 hours and in Mueller-Hinton broth for 6 hours.

Antigen Preparation: Six-hour broth shake cultures were treated with 3% formaldehyde (1 ml/8 ml broth) and centrifuged. The supernatant fluid was poured off, the residue resuspended in 8 ml of 0.15 M NaCl and again centrifuged. The supernatant fluid was decanted and the cells reconstituted to original volume with 0.5% formalin, then stored in a refrigerator at 4° for no longer than a few days.

Vaccine Study: Two-week-old mice (6-8 gm) were immunized at 0 and 7 days, then challenged at 14 days after the original injection. Six immunized and 6 non-immunized mice of the same age were used to determine the LD/50 of the meningococci used to challenge the immunized mice. Log 10 dilutions of a 6-hour shake culture were made and the vaccinated and control mice were challenged with 10^4 through 10^9 dilutions. Deaths were recorded at 24-hour intervals for 4 days. Calculations of LD/50 were made using the Reed-Muench formulae (13).

RESULTS

Table 2 shows the enhancement of pathogenicity in strain X *N. meningitidis* by serial passage in mice. The strain X meningococci killed 3 or 4 mice (brackets) with 10^4 organisms in the 1st passage. Three of 4 mice (brackets) were killed by less than 10 organisms in the 4th passage.

Table 3 shows the enhancement of virulence of 4 other strains of meningococci. A challenge of 10^5 organisms of strain 17 were required to kill 4 mice in the 1st passage. The same number of mice

were killed by 10^4 organisms in the 2nd passage. Two mice died after a challenge of 10^6 organisms with strain 29E in the 1st passage, and a similar number were killed by 10^3 organisms after 1 more passage. The strain Bo (WR) required 10^4 organisms to kill 1 mouse in the 1st passage, but killed 3 of 4 with only 10^3 organisms in the 3rd passage. The virulence of strain 112 was probably enhanced after the initial passage, however, the results with this strain are inconclusive.

Table 4 contains the virulence data of strains Y, RAS 10, and Z, with 3 passages in mice. These results show that the virulence of these groups are not enhanced after 3 passages in mice.

Table 5 shows the results of the study of active immunity to strain X and challenges with homologous and heterlogous organisms. It should be noted that there were no mice challenged with the 10^4 dilutions of organisms in the vaccinated groups. Further, it should be pointed out that the results with 10^5 organisms for the group C challenge of vaccinates and with 10^8 organisms for groups B and C challenge of vaccinates are exceptional in that one would normally expect more deaths at these points. The table shows that more mice died among the control mice than among the vaccinated mice. The LD/50 values were computed from 10^5 through 10^9 dilutions of the organisms. The vaccinated mice challenged with groups B and C survived challenges of 10 and 10.5 LD/50s, respectively. The protective values for strain X and group A were lower than for groups B and C.

DISCUSSION

Enhancement of Virulence: Four of the 8 strains studied showed increased virulence after 2 of 3 passages. Enhancement of virulence probably occurred because the more virulent organisms survived, whereas, the less invasive and/or more easily phagocytized organisms were eliminated. Thus, only the more pathogenic organisms were recovered from the blood.

The other 4 strains showed no increase in virulence. These strains may not have pathogenic potential or may require a larger number of mouse passages to develop pathogenicity.

Several of these 4 strains could not be recovered after each inoculation. This may suggest a need for a change in the bleeding regimen.

Perhaps a larger number of organisms should be used for each inoculum. Miller et al (12) have shown that with a larger number of

organisms in the inoculum, penetration into the blood stream occurs more quickly and the organisms persist much longer.

The part of the study using group C organisms showed minimal protection by LD/50s, but good protection by total deaths, as there were twice as many deaths in the controls as there were among the vaccinates.

Since the enhancement of virulence has occurred with several of these strains, it should be possible to use strains 17 and 29E and group X for immunization and heterologous challenge in active and passive immunization studies.

The wide variation in magnitude of immunologic response may require many more mice per point to get a good distribution in deaths with a variable challenge.

SUMMARY

Meningococcal strains 17, 29E, Bo, 112, Y, RAS 10 and X were passaged consecutively in mice in an attempt to enhance their virulence. Only strains 17, 29E and X increased in virulence. Immunization of mice with whole formalinized cells of strain X failed to afford homologous protection against challenge with strain X. Mice immunized with strain X were protected against heterologous challenges of serological groups A, B, and C of 7, 10, and 20 LD/50s, respectively.

TABLE 1. Nomenclature Comparison Table: Nomenclature Used by Various Investigators and in this Paper, by Investigators at NAMRU#4

Bergey (2)	Slaterus (3,4)	Walter Reed (Evans et al) (5)	Vedros (7)	CDC Proposed (10)	NAMRU#4
A					A
B					B
C					C
D					D
	X			E	X*
	Y	Bo	E	F	Bo** Y*
	Z			G	17*** Z*
	Z'	29E			29E** 112***
					RAS 10***

* Slaterus isolates

** Walter Reed isolates

*** NAMRU#4 isolates from carriers

TABLE 2. Enhancement of Virulence of Group X Meningococci by Serial Passage in Mice

Organism Count Log 10	1	2	3	4
10^0	1**	0	NO COUNT: LABORATORY ERROR	[3]
10^1				
10^2	2	4		3
10^3				
10^4	[3]			3
10^5				
10^6				
10^7				

** Number of mice dead in group of 4 mice.

TABLE 3. Enhancement of Virulence of Strains 17, 29E, Bo(WR) and 112 by Serial Passage in Mice.

	NUMBER OF MOUSE PASSAGES OF THE ORGANISM								
Organism Count	Strain 17		Strain 29E		Strain Bo			Strain 112	
	1**	2	1	2	1	2	3	1	2
10^2	-	-	-	-	0		-	-	-
10^3	-	3*	-	2	-		3	-	3
10^4	2	4	0	2	1		-	2	2
10^5	4	4	0	2	-		1	4	2
10^6	3	-	2	-	-		-	3	-
10^7	-	-	-	-	-		2	-	-

* Number of mice dead of group of four challenged

** Number of passages

TABLE 4. Enhancement of Virulence of Strains Y, RAS 10 and Z by Serial Passage in Mice

	NUMBER OF PASSAGES IN MICE							
Organism Count	Strain Y			Strain RAS 10			Strain Z	
	1	2**	3	1	2	3	1	2
10^0	1*		-	1	-	-	1	-
10^1	-		-	-	-	-	-	1
10^2	1		-	0	-	-	1	-
10^3	-		0	-	-	-	-	1
10^4	0		-	2	-	0	3	-
10^5	-		1	-	-	0	-	2
10^6	-		-	-	-	0	-	-
10^7	-		6	-	-	-	-	-

* Number of mice dead per group of four challenged

** Deaths not shown because bacterial count was unreliable

TABLE 5. Mice Vaccinated with Strain X antigen and Challenged with Groups A, B, C and Strain X Organisms

Organism Dilution (Log 10)	Strain X		Group A		Group B		Group C	
	Con*	Vac**	Con	Vac	Con	Vac	Con	Vac
10^4	2		6		5		6	
10^5	5	4	6	4	5	5	6	1
10^6	3	5	6	5	6	4	6	4
10^7	3	2	6	5	5	4	6	6
10^8	3	2	5	4	4	0	5	0
10^9	2	2	2	1	1	3	1	1
No. Dead***	16	15	25	19	21	16	24	12
LD/50 dilution	7.16	6.88	8.63	7.82	8.22	7.22	8.49	7.20
Protection****		1.91		6.50		10.00		19.50

* Control: non-vaccinated mice
** Vaccinated mice: mice vaccinated with strain X antigen
*** Number dead 10^5 to 10^9, inclusive
**** Protection: expressed in LD/50s

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THE ASSOCIATION OF ORGAN ANTIBODIES WITH PNEUMONIA AND ACUTE RESPIRATORY DISEASE IN NAVY RECRUITS*

Ensign George R. Arends
University of Michigan Medical School

Preceptor: E.A. Edwards
Chief, Immunology Division

"Although there has been much interest recently in the phenomenon of autoimmunity in a variety of diseases, very little investigation has been concerned with pulmonary disease" (4). This is as accurate a statement now as it was in 1963. The subject needs investigation since the reports available are inconclusive and incomplete.

The phenomenon of autoimmunity itself is controversial. While 1 theory holds that the inflammatory and destructive lesions of autoimmune diseases affecting various organs and tissues are due to mutational changes in lymphoid cells or to the appearance of antigenically foreign characters in the cells of target organs (19), another postulates that circulating autoantibody may simply be a response to previous tissue damage and may be a protective, rather than causative factor in disease (12). A third suggestion is that clones of "foreign" lymphocytes grow up from pre-existing lymphocytes producing antibody against specific organs (7). Another proposes that the breakdown of anatomic or physiologic isolation of tissues causes organ-specific antibodies to occur (7). The hapten mechanism of antibody formation postulates a combination between the organ involved and some chemical, bacterial or viral antagonist as the antigenic unit which brings about antibody production (7,12,14). A final proposed mechanism implicates antigenic components of microbial agents as giving rise to antibodies which are cross-reactive with specific tissue constituents (13,14).

Although agreement does not exist on the mechanism of autoantibody production, that antibodies occur against organ systems

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is well substantiated. Tissue antibodies have been demonstrated in both normal animals and in individuals showing no disease state by means of the CF test (14,19).

Of more significance have been reports of organ antibodies associated with disease states (10). Thyroid autoantibodies were shown by CF tests to occur in thyrotoxic patients by Papazola in 1911 (15). Injection of homologous brain tissue into animals has produced circulating antibody and encephalomyeltis (8,15). Auto-spermagglutinins have been shown to be present in the sperm of patients with granulatomous orchitis (6), and have been implicated in infertility in men (16). Glomerular-basement membrane has been used as an antigen. Antibodies form against it and cause nephritis (9). Adrenal tissue in saline has been used in CF tests run on patients with idiopathic Addison's Disease to demonstrate serum antibodies (1). The enumeration of possible autoimmune diseases could be continued further, for the list of conditions and organ-specific antibodies is already extensive and grows longer each year.

Data substantiating the occurence of autoantibodies in diseases of the lung are contradictory. While some have shown that antibodies do occur against lung tissue in patients with chronic diseases, such as emphysema, pulmonary tuberculosis, and bronchiogenic carcinoma, and even against specific lung constituents (elastin, reticulin, and collagen)(2,3,12), others have been unable to demonstrate significant lung antibodies associated with disease (20), or have shown antibodies which react with several tissue types, lung included (11,18). In primary atypical pneumonia caused by Eaton's agent (a mycoplasma), lung-specific antibody has been demonstrated by CF (19). It occurs during the convalescent period of the disease. Little data are available concerning the topic of lung disease and autoimmunity.

The purpose of this study is (1) to determine the normal organ antibody level for lung in recruits entering Great Lakes for training, (2) to follow the development of lung antibodies during training and correlate this with their vaccination schedules, (3) to study the relationship of organ antibodies to different types of pneumonia, and (4) to show whether lung antibodies occurring in disease are organ-specific.

MATERIALS AND METHODS

Antigens were prepared by the method of Burrell et al (5) with these modifications: tissues were cut into 1 cm squares and washed in veronal-buffered saline (VBS) in a Psychotherm Incubator

Shaker at 200 RPM, 25°C for 3 days. To prevent bacterial growth, 1 ml of 1% merthiolate per 100 ml VBS was added. The supernatant fluid was discarded. The washed tissue was then cooled in an ice bath during maceration in a Sorval Omni-Mixer for 10 minutes at 10,000 RPM. After blending, 3 or 5 volumes VBS were added to 1 volume of tissue to make up the tissue suspensions. These suspensions were then placed in an ice bath on a Sonifier Cell Disruptor for 5 minutes on setting 5 at 100 watts. The solutions were then stirred on a Bell Stir magnetic stirrer for 12 hours in a refrigerated unit at 5°C. Next, they were centrifuged in a Sorval Super Speed RC-2 at 7.500 RPM at 0°C for 30 minutes. The supernatant antigen was collected and stored at 5°C. Certain antigens were further purified by using gel filtration and a Sephadex #100 column, 17 cm in length.

Specific antigens (Ag) employed in testing are antigens A, B, and C prepared at a 1:6 suspension, as described above, from the normal lung tissue of a female between 20 and 30 years old. Three ml of antigen A were further purified by gel filtration. Twelve 3-ml fractions were collected. Fraction A Ag #7, diluted 1:3 during filtration, was used extensively in testing.

Mixed lung tissue consisting of alveolae, blood vessels, bronchi, and pleura from the normal lungs of a 71-year-old man made up Ag 71 #2. It was prepared as a 1:6 tissue suspension.

From a 61-year-old woman, with a history of chronic asthma and frequent respiratory distress, lung, kidney, and liver antigens at 1:3 suspensions were prepared. The lung antigen Ag 61-L consisted of mixed lung tissue.

The determination of antigenic potency was made by a straight line titration. Four units of antibody were dropped in each dilution of antigen. The highest dilution of antigen showing a 4+ fixation (no hemolysis) was read as 1 CF unit. Four CF units of antigen were used throughout the test series.

Anti-lung antiserum was prepared by giving a rabbit 4 intramuscular depot injections containing a total mixture of 5 ml tissue suspended in 10 ml of Freund's complete adjuvant. After 14 days the rabbit was bled by cardiac puncture, the serum separated, and stored frozen.

Human serum used in testing came from a variety of recruit populations. The delayed vaccination company (also control company) was the July 1967 surveillance company. They received the standard series of vaccinations: polio, smallpox, diphtheria,

tetanus, typhoid, paratyphoid, yellow fever, typhus and cholera, in the normal sequence. However, flu vaccinations, bicillin, and sulfa prophylaxis were withheld. Serologic surveillance showed an average of less than 10% of the company infected by adenovirus, influenza, and meningococcus.

The normal vaccination company was a winter company involved in the amantadine influenza vaccine protection study. They received the standard series of vaccinations plus bicillin and sulfa prophylaxis. In addition, they received, by random selection, combinations of amantadine, placebo, influenza vaccine and a saline placebo. Serologic surveillance showed an average of greater than 50% of the company infected by adenovirus, influenza and meningococcus.

Other groups consisted of serum from random hospital admittances for pneumonia of various types, and serum from men in a live adenovirus vaccine study hospitalized for pneumonia. A final population consisted of serum from 50 consecutive recruit admittances for pneumonia to Great Lakes Naval Hospital during October 1967. The ages of the men in all populations ranged between 17 and 25 years.

The CF test, as adapted to microtechnique by Sever (17), was the primary method used to demonstrate organ antibodies and is briefly described as follows: .025 ml serum was titrated using VBS as the diluent. To this, .025 ml of antigen containing 4 CF units were added along with .05 ml complement containing 2 exact units. The system was mixed and incubated at 5°C for 18 hours. (Incubation at 18 hours produced an 8-fold increase in titer over tests incubated at 37°C for 1 hour.) After incubation, the system was warmed and .050 ml of a 2% solution of activated sheep erythrocytes were added. The plates were covered, shaken and incubated at 37°C until the complement control gave a 4+ fixation in the 4th antigen control, and positive serum control were included in each test sequence. Titers were read as the highest dilution giving a 4+ fixation.

Serum was inactivated in a water bath at 56°C for 1/2 hour the 1st time it was tested, and for 10 minutes on each subsequent test. Because of the frequent freezing and thawing of the serum samples, each serum was titrated out in testing its anti-complementary properties.

RESULTS

The results tabulated on Table 1 suggest that recruits receiving standard vaccinations have no detectable lung antibody,

but after receiving bicillin and sulfa prophylaxis, and flu vaccinations, a statistically important number showed an increase in lung antibody. Why these medications should cause lung antibody to be formed is not known. One possible difference, other than the medications received, which could have influenced the results, was the percent infection per company. The recruits receiving standard vaccinations were a summer company showing less than 10% infection by serology, while those receiving medication were a winter company showing greater than 50% infection by serology.

To state that organ antibodies to lung do occur in young men with clinically positive cases of pneumonia from the data collected in Table 2 is not possible. The titers recorded as any rise or any fall are comprised, primarily of 1 tube (2-fold) changes in titers. These fall within the error of the test, and are not statistically valid changes, but only represent trends. Thus, even though pneumonia cases with positive X-rays do show a statistically valid number of titer rises indicating the presence of lung antibody, this can be considered only as a trend. Lobar pneumonia, pneumonitis, and pneumonia of no confirmed etiology show no statistically valid trends confirming the presence of lung antibody.

When the group of positive X-ray pneumonia sera is compared in Table 3 by statistically significant 4-fold titer rises, the validity of the trend shown in Table 2 is strengthened. Ag 61-L confirms the presence of lung antigen in 5 of 53 men showing positive X-rays, a comparison having 50% statistical reliability. Tests with A Ag #7 show the presence of lung antibody in 14 of 53 men with positive X-rays, a comparison of greater than 99% statistical reliability.

When data from all 148 pneumonia cases studies (shown in Table 4) is combined to increase the sample size, rises of 1 tube to Ag 61-L occur in 28 of 148 men. These data suggesting the presence of lung antibody have greater than 99% statistical reliability. However, proof of the presence of lung antibody based on statistically significant titer changes was not obtained. When the 19 four-fold titer rises of 148 men studied were analyzed, no statistical credence could be given in support of this data as proof of the presence of lung antibody.

The correlation (Table 5) of serologic response to lung and kidney antigen by individuals having positive cultures for *Mycoplasma pneumonia* is not statistically significant. It is of interest to note, however, that positive isolations of *M. pneumonia*

show 4-fold rises in CF titer in only 2 of 7 cases, and that 3 CF rises occur in serum with negative isolation data for M. pneumonia.

Perspective on these results is gained when one notes that antigen prepared from any of the 3 available lungs is of similar quality. The maximum titer obtained from any of the human sera tested was 64. These results point to a very weak human-serologic response.

Titers obtained from the rabbit controls consistently ran 32 to 64, and reached 512 maximums in several of the antigen titrations. The rabbit anti-lung antiserum, however, also gave a 128 titer to liver antigen and a 512 titer to kidney antigen. This suggests production of a non-specific antibody by the rabbit which is reactive with several organ antigens.

DISCUSSION

The lack of organ specificity displayed by rabbit lung antibody in this experiment has previously been reported by Gajdusek (10). When the evidence by Turner-Warwick (20), failing to show the occurrence of organ-specific lung antibodies in man is considered, one questions exactly what the antibody is in the serum samples which react with "lung antigen."

Although Muschel (14) has reported finding tissue antibodies in animals by means of CF tests, this study has been unable to show with statistical confirmation the presence of antibody to lung tissue in young individuals, healthy or suffering from pneumonia. Gajdusek (10) reporting on autoimmune diseases has noted that "positive reactions were rare in sera from normal subjects." In his study using liver antigen, Gajdusek does report finding high titers to some individuals' sera, but qualifies these as being found in association with "a few pathologic conditions."

The work of Hennes et al (12) and of Balchum et al (3) has shown no significant lung antibody in normal individuals, but has confirmed the presence of significant antibody to lung tissue in patients with chronic lung diseases (obstructive emphysema, tuberculosis with cavitation, and bronchiogenic carcinoma).

The crux of lung antibody production, as correlated with disease states, seems to lie in the acute or chronic nature of the disease. Respiratory diseases of acute onset seem to lack lung antibodies, while chronic pulmonary diseases show increased titers to lung antibody.

SUMMARY

(1) Recruits receiving standard vaccinations show no detectable lung antibodies. (2) However, after receiving influenza vaccinations, bicillin and sulfa prophylaxis, or after extensive serological infection by adenovirus, influenza, and meningococcus, evidence points to a statistically significant rise in lung antibody. (3) Limited data suggested production of weak lung antibody in acute lower respiratory disease, although this finding was statistically inconclusive. (4) Limited data suggested that human antigen did not cross-react with kidney antigen in the context in which this was studied.

Tables 1 through 5 summarize the series of tests run. Each rise recorded is a 1 or 2 tube rise. Of those sera showing rises, one in three was anti-complementary.

In Chi square analysis of the data, Yate's correction was applied where necessary.

TABLE 1. Comparison of Companies Receiving Routine and Delayed Vaccinations for Serological Evidence of Anti-lung Antibody

	Companies Tested Against Antigen 61-L at 1:30	
Titer	Normal Vaccinations*	Delayed Vaccinations**
No Change	57	65
Any Rise	21	2
Any Fall	1	0
Totals	79	67

Chi Square = 13.866 $p < .005$

* This company was in the amantadine-influenza vaccine protection study. No differences by injection groupings were noted.

** This company did not receive influenza or adenovirus vaccine, nor bicillin and sulfa prophylaxis.

TABLE 2. Serological Response to Lung Tissue Antigen by Recruits Admitted to Great Lakes Naval Hospital with Lower Respiratory Complaints

Groups Tested Against Ag-61-L at 1:30

Titer	Pneumonia Positive X-ray	Lobar Pneumonia	Pneumonitis	Pneumonia of No Confirmed Etiology	Control Company
No Change	26	14	5	13	65
Any Rise	20	3	2	2	2
Any Fall	7	1	0	5	0
	$x^2 = 32.766$ $p < 0.005$	$x^2 = 3.55$ $0.25 < p < 0.10$	$x^2 =$ Not Significant	$x^2 =$ NS	

Titer	Pneumonia P Positive X-ray	Lobar Pneumonia	Pneumonitis	Pneumonia of No Confirmed Etiology	Control Company
No Change	24	16	6	18	65
Any Rise	22	2	1	3	2
Any Fall	7	0	0	0	0
	$x^2 = 34.672$ $p < 0.005$	$x^2 =$ NS	$x^2 =$ NA	$x^2 =$ NS	

TABLE 3. Four-Fold Titer Changes

	Pneumonia Positive X-ray Group		
Titer	Ag-61-L at 1:30	A-Ag #7 at 1:20	Control Company
No Change	48	35	65
Four-fold Rise	5	14	2
Four-fold Fall	0	4	0
	$Chi^2 = 1.647$ $0.5 > p > 0.25$	$Chi^2 = 16.359$ $p < 0.005$	

TABLE 4. Comparison of 2-Fold Titer Changes with Significant Four-Fold Titer Changes in Combined Pneumonia Cases

	Groups Tested Against Ag 61-L	
Titer	Combined Pneumonias	Control
No Change	107	65
Two-fold Rise	28	2
Two-Fold Fall	13	0
Total	148	67

Chi Square = 15.043 $p < 0.005$

	Groups Tested Against Ag 61-L	
Titer	Combined Pneumonias	Control
No Change	124	65
Four-fold Rise	19	2
Four-fold Fall	5	0
Total	148	67

Chi Square = 5.451 $0.1 > p > 0.05$

TABLE 5. Correlation of the Serologic Response to Lung and Kidney Antigen with the Isolation of and the Complement Fixation Rises to Mycoplasma Pneumoniae in Fifty Random Pneumonia Admittances to Great Lakes Naval Hospital

	Mycoplasma pneumonia				
	Positive Isolations		Negative Isolations		
Titer	CF Rise	No CF Rise	CF Rise	No CF Rise	Total
Lung-No Change	0	5	3	40	48
Lung-Rise	2	0	0	0	2
Kidney-No Change	2	5	3	39	49
Kidney-Rise	0	0	0	1*	1

*Showed No Response to Lung Antigen

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PRELIMINARY REPORT ON INTERFERON-LIKE RESPONSES OF NAVAL RECRUITS FOLLOWING YELLOW FEVER AND OTHER IMMUNIZATIONS*

Ensign Robert Hartmann
University of Illinois, School of Medicine

Preceptors: J.A. Gray and M.J. Rosenbaum, Ph.D.
Virology Division

Interferon (IF) is a non-specific antiviral substance which can be derived from cells stimulated by a variety of biologicals (1-4). Among these inducers are active and inactive virus preparations, as well as somatic and intracellular bacterial products (5). Many such substances are given to humans for immunizations against epidemic agents. Wheelock (2) has demonstrated that yellow fever (YF) vaccine was capable of inducing detectable levels of IF in young adults. Measles (3) and influenza virus (6) have also been shown to induce IF in humans. Several of the routine inoculations of military recruits contain substances which are known inducers of IF. These include influenza, poliovirus, smallpox, YF, and typhoid vaccines. Other preparations such as tetanus, diphtheria, cholera, and typhus vaccines also are likely to stimulate IF in recipients.

To determine whether all or any of these materials are effective in the production of IF, a study of such responses in Naval recruits was initiated.

The specific objectives of this study were as follows:

1. To determine the magnitude and duration of IF production after YF or other routine immunizations.

2. To compare IF responses in vascular cells and fluids with avascular secretions, such as saliva and urine.

3. To determine whether YF vaccine renders white blood cells from vaccinated donors more reactive to IF induction than cells from control subjects.

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*From Research Project No. MR 005.09-0084, Bureau of Medicine and Surgery, Navy Department, Washington, D.C.

MATERIALS AND METHODS

Because of the difficulty in studying the responses to all of the treatments received by recruits in training, only 1, the YF vaccine, received detailed attention. The relationship of the other vaccines and treatments were only observed insofar as how they might have affected the YF responses.

Subjects and Treatments: The design of the study, specimen collection dates, variety of specimens obtained and the time of immunizations and other prophylactic measures are shown in Table 1.

The study population consisted of a company of recruits (80 men) who were beginning training. The company was divided into 2 groups of 40 men each and designated as Blue or Gold. Yellow fever vaccine was administered to only 1 group at a time. This was done to provide a control group for those receiving vaccine at either instance. The Blue subjects received their YF vaccine during the second week of training, the Gold in the fourth week. All other treatments were administered to both groups at the times indicated on the schedule.

Specimens and Tests: Samples of blood, urine, and saliva were obtained on the days indicated in Table 1. Blood specimens were obtained with and without heparin (phenol-free). Serum from clotted bloods were removed and stored at -10°C until tested. White blood cells were obtained from plasma and infected with 10^7 EID_{50} of Newcastle disease virus using Wheelock's techniques shown in Fig. 1 (7).

Saliva specimens were obtained from subjects after chewing of wax gums and stored at -10°C. Early morning urine voids were collected from the respective groups at the indicated intervals (Table 1). Each day's collection was pooled according to group and stored at -10°C.

Interferon assays were carried out by the method of Wheelock and are described in Fig. 2 (7).

DISCUSSION OF RESULTS

The following preliminary data was derived from an analysis of IF assays of 728 serum specimens. This is approximately 70% of the total number collected. Because of the large number of specimens screening procedures were employed. Serum was diluted to 1:10, 1:100 and 1:1000. Interferon was considered present in any

dilution which showed 50% greater inhibition of 10,000 TIC_{50} of Sindbis virus. The data shown in Figure 3 were arranged according to experimental group (Blue or Gold) and percent of men in either group with serum IF titers of 1:10 or greater at each of 13 sampling times. Also shown (Fig. 3) are the times of routine or experimental (YF) immunizations and their relationship to the fluctuations in percent of men with IF.

It is evident that the 2 groups are different in their responses to YF vaccine. The Blue group, who received the immunization during the early part of training, show only a minimal increase in the percent of men IF positive during the week following vaccination. According to Wheelock, maximal responses occurred 6 days after YF inoculation (2). Such increases in responses and agreement with Wheelock's data occurred in men in the Gold group, but not in the Blue group.

It can be observed that the Blue group was immunized with other vaccines during their response period, and these may have either taken up circulating IF or repressed its production. Similar phenomenon has been observed by Youngner with IF responses to bacterial endotoxin (8). In his studies, a state of IF hyporeactivity was induced when mice were immunized after 2 successive doses of endotoxin within 2 weeks.

The data also suggest that the other vaccines may be stimulating IF production. A small increase in both groups in the percent of men with IF titers is shown after the initial poliovirus, influenza, and diphtheria-tetanus vaccination in the preliminary and final weeks.

These increased responses, including those to YF vaccine, are transient and most regress to lower levels within 2 weeks after immunization. The data obtained thus far indicate that certain non-specific factors in serum, assumed to be IF, are enhanced by vaccination with YF vaccine. Superimposition of other vaccines appear to repress its production or deplete what is present. Another alternative hypothesis is that the time of immunization may be important in respect to the IF response. This may be due to host factors which are affected by the stress and rigors of the recruit training. These findings are compatible with those of Pierce et al (9), who observed that delay of routine inoculations until the latter half of training was associated with a 20% reduction in acute respiratory disease and pneumonia.

These studies will be continued to assay the remaining serum specimen, as well as the other types which have been collected. Furthermore, it would be interesting to determine whether or not the failure to show IF responses in the Blue group was due to inactivation of YF virus by IF produced by the previous immunization with influenza or poliovirus or diphtheria-tetanus vaccines.

SUMMARY

Preliminary results from a study of 80 Navy recruits immunized with YF and other vaccines suggested that interferon-like substances are induced by such procedures.

The data also inferred that IF responses may be suppressed or available IF depleted if sequential vaccinations are made within intervals of less than 2 weeks.

ACKNOWLEDGEMENT

I wish to express my appreciation to Dr. Rosenbaum tor his guidance during this project and also to the members of the department of virology for their help. In particular, I wish to thank Chief Donald Autry, Mrs. Elizabeth Bannon and Mr. Frank Grockis for their help in the processing of the samples. Lastly, I would like to thank Dr. Johnson, Chief Koonce and the members of the field lab for their aid in the many collections.

TABLE 1. Schedule for Yellow Fever Vaccination and Specimen Collection

Subjects	Treatment or Specimens		Week of Training: I		II				III	IV				V	VI	VII
			Day relative to YF vaccination													
		-12	-7	-4	0	1	2	4	7	14	15	16	18	21	28	35
Blue (40 men)	YF vaccine				X											
	Blood		X		X	X	X	X	X	X			X	X	X	X
	Urine			X	X	X	X	X	X	X			X	X	X	X
	Saliva			X	X	X	X	X	X	X			X	X	X	X
		-26	-21	-18	-14	-13	-12	-10	-7	0	1	2	4	7	14	21
Gold (40 men)	YF vaccine									X						
	Blood		X		X			X	X	X	X	X	X	X	X	X
	Urine			X	X			X	X	X	X	X	X	X	X	X
	Saliva			X	X			X	X	X	X	X	X	X	X	X
Routine inoculations		Flu Polio Diphtheria-Tetanus (D-T)			Bicillin	Smallpox Typhus Cholera Typhoid Paratyphoid (T-P)									D-T	Polio T-P Typhus Cholera

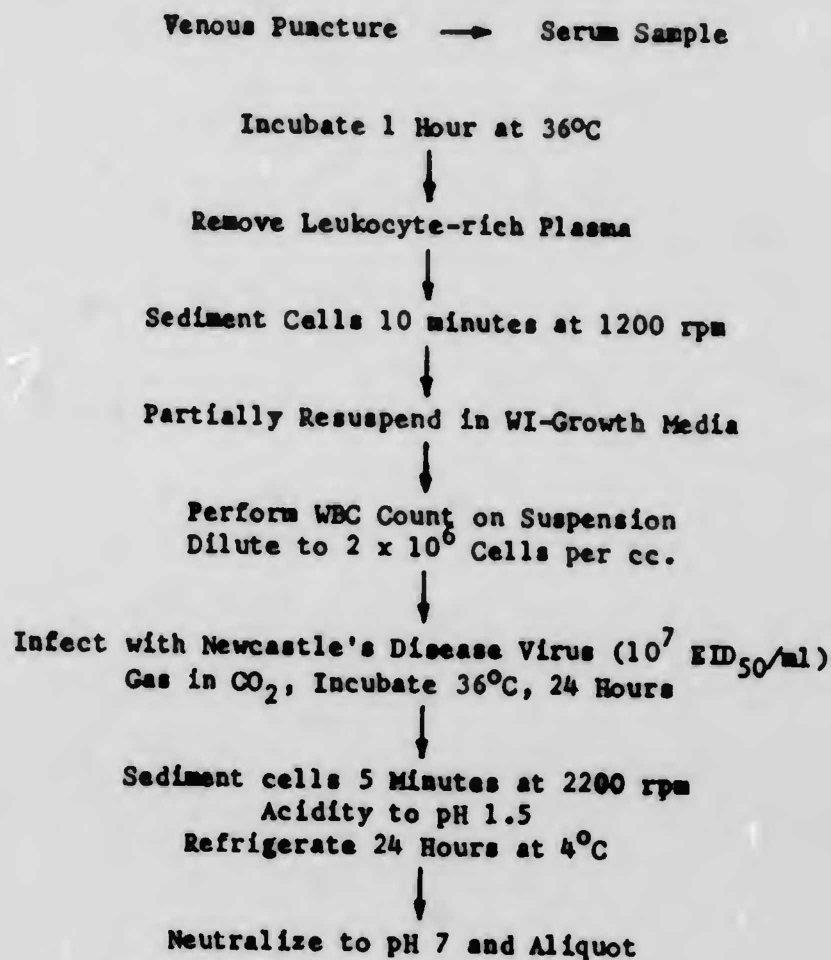

Fig. 1. Procedure for induction of interferon from white blood cell cultures.

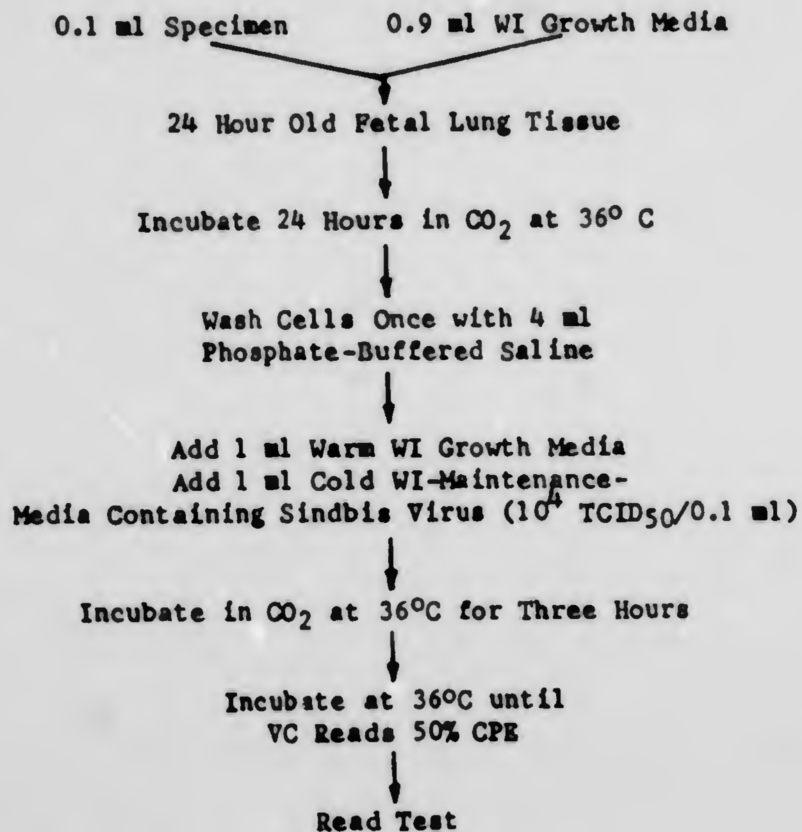

Fig. 2. Procedure for assay of interferon.

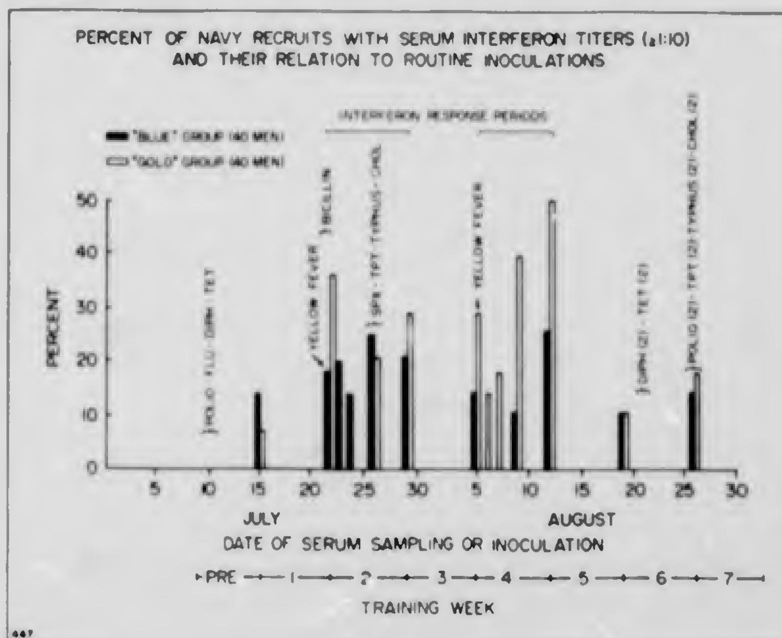

Figure 3

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NEW MEDIA FOR ISOLATION OF MYCOPLASMA*

Ensign Edward W. Millunchick
University of Iowa College of Medicine

Preceptor: W.H. Kraybill
Chief, Mycoplasma Research Division

Mycoplasmas are notorious for their slow growth and, in some cases, difficulty of isolation on artificial media (1). Efforts have been made by Ogata et al (2) and others to compound new media for improved myooplasma growth and isolation. They studied the effect of substituting calf serum vs horse serum as a nutrient base for growth of *Mycoplasma pnemoniae*, *M. pharyngis*, and *M. fermentans*, as well as a tissue culture (TC) isolate, which resembles *M. hyorhinis*.

A second approach stems from the fact that mycoplasmas grow as contaminants in TC (5). The present study compares media, comprised of various TC constituents incorporated into an agar base, with standard PPLO medium for their ability to support growth of mycoplasma of human and TC origin. This may be helpful for isolating aberrant mycoplasma strains occurring in TC, since Sullivan (3) was able to isolate a mycoplasma from TC in cell line growth media, but not on conventional PPLO agar. In addition, the effects of yeast extract, horse and calf serum, and of TC cells on mycoplasma growth were observed. The effect of yeast extract as a growth factor is described by Ogata et al (2) and Kurzepa (12). Tissue culture cells may be the source of some necessary nutrients for mycoplasma growth evident in TC cells, but not on PPLO agar.

MATERIALS AND METHODS

The reagents used are outlined below. Eagle's Basal Medium (EBM)(8) was incorporated into agar by the methods outlined below. The agar base was in a concentration of 0.9% for all media. Serum from newborn calves, lacking gamma globulin, was used as a nutrient since this is a reagent often used in TC. Gamma globulin-free horse serum was also used as a nutrient in companion plates, since studies have

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*From Research Project No. MR 005-09-0024, Bureau of Medicine and Surgery, Navy Department, Washington, D.C.

shown that it is the best serum enrichment for most mycoplasma. The yeast extract enrichment was prepared as outlined by Crawford (9). HEp TC cells were used at a concentration of 2-300,000 cells per ml. Earle's Balanced Salt Solution (EBSS) was used as a solvent for all TC plate materials. All reagents were adjusted to pH 7.0 before mixing. Penicillin 2,000,000 units, amphotericin B (Fungizone) 5 mg /l and thallium acetate 0.5 gm/l were added to inhibit growth of contaminant microorganisms. The plates were prepared using reagents in the proportions shown in Table 1.

Standard Plate Preparation: The procedure for the preparation of standard media (5) used in control plates is outlined in Crawford's manual, page 14 (9). Variations in its preparation were: 1) type of serum used, 2) pH of reagents, and 3) presence or omission of methylene blue.

Tissue Culture Plate Preparation:

1) The agar was dissolved and boiled in EBSS. The pH was adjusted to pH 7.0 with 1N NaOH, and autoclaved, then stored at 4°C in a refrigerator.

2) The horse or calf serum was apportioned to a 4-ounce prescription bottle to which all other reagents were added prior to adding the agar mixture. This included yeast, cells, additional EBSS and basic EBM solution, as outlined in Table 1. (The EBM reagents were mixed to a concentration of 2X so that when added to the agar mixture the final volume would be 1X, which was to be 65% of the entire media.)

3) The plates were poured, labeled, and stored in a refrigerator at 4°C for 4 days before use.

Laboratory strains of M. pneumoniae (M-52), M. pharyngis (CD5), M. fermentans (PG18) and the TC isolate (1465) were inoculated onto the plates at dilutions of 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} in 0.1 ml amounts. Earle's Balanced Salt Solution was used as a diluent. The plates were dried, then incubated anaerobically at 36° in 5% CO_2-95% N_2 for 14 days.

Readings were taken at 3, 7 and 14 days, using a standard dissecting microscope at 40X magnification. When necessary, confirmation of mycoplasma colony growth was accomplished under 100X magnification of a light microscope. Readings of more than 300 were considered too numerous to count. Readings less than 30 were considered too few for accuracy of observations. The only readings considered in the data are the final readings, since we are mainly interested in total growth capabilities.

RESULTS

Table 2 shows the 10 media and 4 organisms tested, together with the ability of the media to support growth. Medium 5 is standard Hayflick PPLO agar. Medium A contains calf serum, medium B contains horse serum. Figure 1 shows *M. pneumoniae*; Figure 2, *M. fermentans*; Figure 3, *M. pharyngis*; and Figure 4, the TC isolate, demonstrating a comparison of growth capabilities of an organism upon several media. Figures 5, 6 and 7 show media A5, B2 and B5, respectively, and their capacity to support growth of the 4 organisms tested.

Also of note is the maximum growth point for each organism on each individual media which supported growth (Table 3). (Maximum growth point is the concentration of broth inoculum at which growth of approximately 150 colonies should be expected; it is similar to the LD/50 point of Reed-Muench.)

DISCUSSION

In most cases, as can be ascertained from Table 2, the media prepared did not support growth. In media A1, A3, B1 and B3, the most likely explanation is the lack of yeast (2). Table 2 illustrates some possibility of using medium A2 to selectively isolate *M. fermentans* and medium B4 to selectively isolate TC strains.

Table 3 may be used to help evaluate the growth capabilities of the media tested. For *M. pneumoniae*, medium B5 seems to support growth of a more dilute solution. However, medium A5 is sufficiently close that further investigation of calf vs horse serum with this particular organism is in order.

M. fermentans grew best on A5 and B5 media, B5 having slightly better growth capabilities. Media A2 and B2 are almost a full order of magnitude below A5 and B5, indicating that the TC medium would probably not be successful. However, since *M. fermentans* was the only organism that grew on A2, this medium may be useful for isolating *M. fermentans*.

M. pharyngis grew slightly better in medium A5 than in medium B5, indicating that calf serum may be useful for growing this organism.

The TC isolate grew best on B5 medium. A5 medium was a close second best. Medium B4 supported growth of only the TC isolate and may, therefore, be useful in further attempts to isolate mycoplasma believed to contaminate TC cell strains.

SUMMARY

An attempt was made to devise new media in an attempt to obtain improved growth and isolation capabilities of the human strains of mycoplasma. None of the new media tested proved significantly more efficient for supporting growth than existing media, although, differential capabilities of media which might prove useful, as a part of isolation techniques, were elucidated.

Prospective studies to compare attempts at isolation or organisms from patients with these variants of existing media and standard media might prove of value.

TABLE 1. Composition of Special Media for Incorporation of EBME Tissue Culture Medium into Agar

Medium #	ADDITIVES (%)					
	EBME In Agar	Serum	Cells	Yeast	Add'l Earles Salt Sol.	PPLO Agar
1	65	20	0	0	15	0
2	65	20	0	10	5	0
3	65	20	5	0	10	0
4	65	20	5	10	0	0
5	0	20	0	10	0	70

TABLE 2. Media Which Supported Growth

MEDIA*	M. pneumoniae	M. fermentans	M. pharyngis	TC ISOLATE
A1	-	-	-	-
A2	-	+	-	-
A3	-	-	-	-
A4	-	-	-	-
A5	+	+	+	+
B1	-	-	-	-
B2	+	+	-	+
B3	-	-	-	-
B4	-	-	-	+
B5	+	+	+	+

* Media A contain calf serum;
  Media B contain horse serum

TABLE 3. Maximum Growth Point* of Various Media

M. pneumoniae	INOCULUM CONCENTRATION
MEDIA A5	$10^{-4.4809}$
B2	$10^{-4.2482}$
B5	$10^{-4.5444}$
M. fermentans	
MEDIA A2	$10^{-3.5000}$
A5	$10^{-4.6173}$
B2	$10^{-3.7874}$
B5	$10^{-4.6396}$
M. pharyngis	
MEDIA A5	$10^{-2.5988}$
B5	$10^{-2.5682}$
TC ISOLATE	
MEDIA A5	$10^{-2.6289}$
B2	$10^{-2.5415}$
B4	$10^{-2.5814}$
B5	$10^{-2.9434}$

* Maximum growth point is the concentration of broth inoculum at which growth of approximately 150 colonies could be expected.

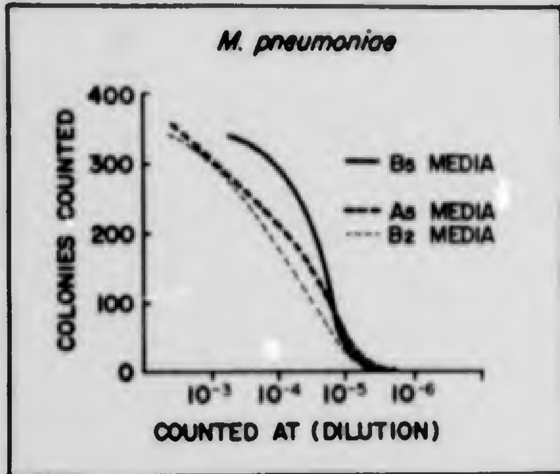

Figure 1

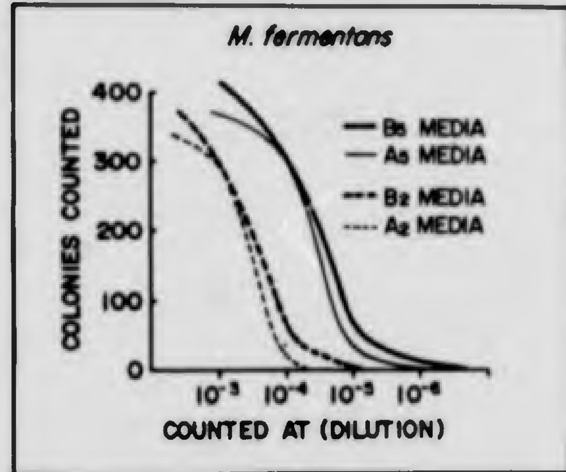

Figure 2

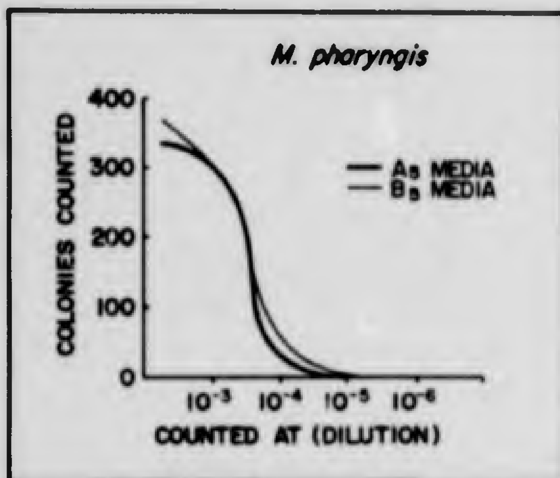

Figure 3

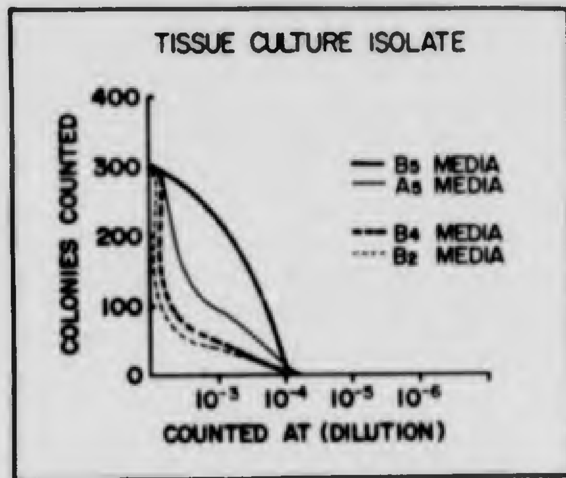

Figure 4

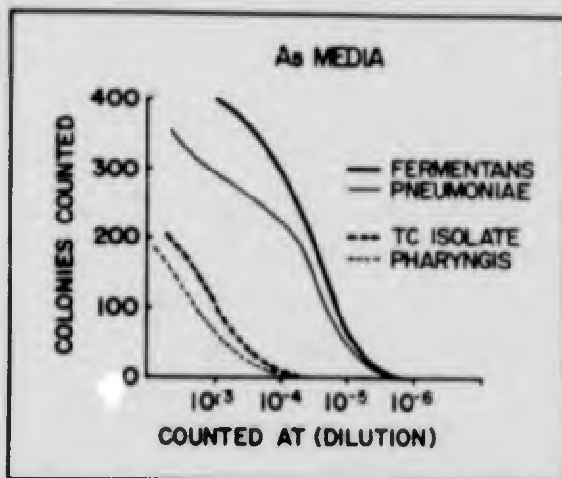

Figure 5

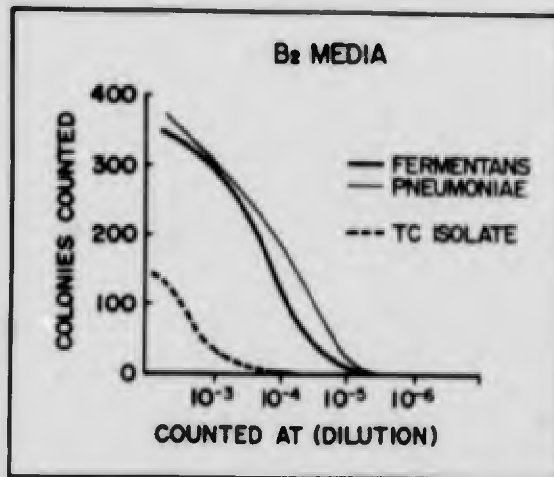

Figure 6

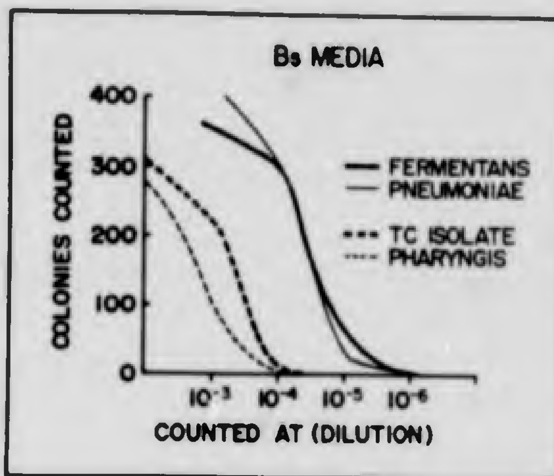

Figure 7

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IMMUNOGLOBULIN SPECIFICITY OF ADENOVIRUS TYPE 4 ANTIGEN*

Ensign Thomas W. Schueppert
Marquette Medical School

Preceptor: E. A. Edwards
Chief, Immunology Division

In 1956, it was shown that infection with adenovirus produced a rise in the level of circulating neutralizing antibody. Antibody levels following infection remained elevated and were associated with resistance to re-infection (1). A similar antibody response was elicited by immunization with inactivated (2) or live adenovirus type 4 enteric-coated tablet (3). Because of the interest in classes of immunoglobulins associated with antibody to a variety of antigens, the question was raised as to the class of immunoglobulin that results from natural infection and by immunizations.

Initial work in this area was done by Lehrich, Kasel and Rossen (4) who used Sephadex G-200 to separate the immunoglobulins and related neutralizing antibody to specific classes of immunoglobulin. Also, work by Bellanti, Artenstein and Buescher has indicated that the predominant virus neutralizing activity in serum was found in association with the 7S IgG immunoglobulin (5). The purpose of this study was to identify the classes of immunoglobulin associated with precipitating antibody by immunoelectrophoresis and immunodiffusion. No attempt was made to relate precipitating antibody with neutralizing antibody.

MATERIALS AND METHODS

Immunodiffusion Slides: One percent agar in .015M NaCl(saline) or in .1M barbital buffer pH 8.6 was used for immunodiffusion and immunoelectrophoresis, respectively. Merthiolate was added to a concentration of 1:10,000 as a preservative.

Slides were prepared as described by Crowle (6). Briefly, this consisted of pipetting a thin layer of hot agar over an alcohol

*From Research Project No. MR 005-09-0087B, Bureau of Medicine and Surgery, Navy Department, Washington, D.C.

cleaned slide, permitting it to dry at room temperature for at least 2 hours or overnight in a dessicator jar when possible. Approximately 3 ml of agar was then applied over the slide which made a layer of agar 2 mm thick. After the agar had solidified, appropriate wells were made in the agar for either double diffusion or immunoelectrophoresis.

Antigen: Electrophoresis was carried out for 3 hours using 3 Ma constant current for each slide. A tracer dye was used to indicate progress of electrophoresis.

Immune Sera:

Human--Sera (acute and convalescent) collected from recruits who were known to have had adenovirus type 4 infections were used.

Animal--Precipitates consisting of adenovirus type 4 antigens and human anti-adenovirus type 4 antibody formed in double immunodiffusion plates were sliced from the plates with a sharp blade, washed 72 hours in 4 changes of excess saline and injected into rabbits with Freund's Adjuvant in the following schedule:

day 1 - 2.5 ml subcutaneous injection of complex emulsed in complete Freund's Adjuvant

2.5 ml intramuscular injection of complex with FA

.5 ml intravenous injection of complex

days 3, 5, 7 and 10 -

.2 ml intravenous injection of complex

Selective Absorption of Immunoglobulin Type: Goat antiserum to human IgA and IgM and horse antihuman IgG (Hyland Laboratories, Lots #5211C2, 8212D005A1 and 8401D001A1, respectively) were used to absorb the specific immunoglobulin class from human serum containing adenovirus type 4 antibody. The hyperimmune antiserum was used at a ratio of 5:1 to the serum containing adenovirus type 4 antibody. Absorptions were carried out at time intervals of 1, 24, 48, 96 and 124 hours. At the end of each absorption time, the mixture was centrifuged at 10,000 rpm in an RC-2 refrigerated centrifuge. The temperature was maintained at 2-8°C. Supernatants of each were used in the immunodiffusion tests for presence of specific immunoglobulin class and specific antibody precipitin activity.

RESULTS AND DISCUSSION

The general pattern for the double immunodiffusion is shown in Figure 1 and the immunoelectrophoresis in Figure 2. From these 2 patterns, it appears that human antibody to adenovirus type 4 does not identify with either IgG, IgM or IgA.

Data from absorption experiments demonstrate complete absorption of IgM and IgA with no reduction in anti-adenovirus type 4 titer. This is conclusive evidence that neither of these human immunoglobulins are related to antibody associated with the precipitin reaction. However, absorption of the serum with rabbit anti-IgG, reduce the IgG level of the serum and also reduced the anti-adenovirus type 4 antibody level. This gives support to the idea that human antibody to adenovirus type 4 is related to the immunoglobulin IgG class. No attempt was made to identify other immunoglobulin classes, such as IgD and IgE.

Rabbits which were immunized with the washed human antibody /adenovirus type 4 complex show antibody against both human immunoglobulin and adenovirus type 4. Precipitin studies using rabbit antihuman serum/adenovirus type 4 complex suggest that more than one serum factor may participate in the precipitin reaction, although there is no line of identity between the rabbit antihuman serum fractions and IgM or IgA. Complete identification of the other serum fractions was not made. Immunodiffusion studies on the antigen-antibody complex was not made prior to immunizing rabbits. The multiple antibody against human-serum fraction may have been due to inadequate washing of the antigen-antibody precipitate before animal immunizations.

SUMMARY

Human antibody to adenovirus type-4 precipitates with adenovirus type 4 in immunodiffusion plates. While the antibody fails to show lines of identity with IgG, IgM and IgA, absorption studies suggest that the antibody belongs to the IgG class of immunoglobulin.

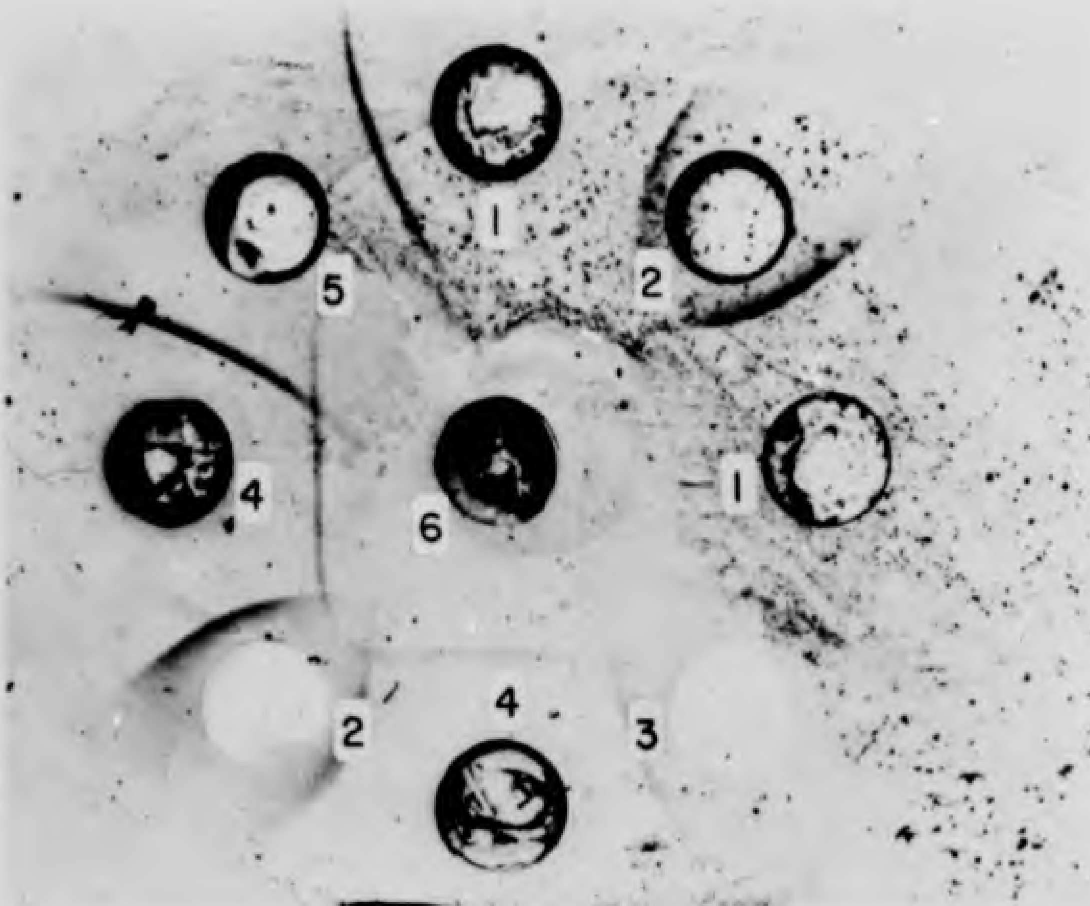

Figure 1. Double immonodiffusion showing lack of identity between antibody precipitating with adenovirus type 4 and anti-immunoglobulin IgG, IgA and IgM. (1) Acute human serum. (2) Anti-human IgG. (3) Anti-human IgA. (4) Convalescent human anti-adenovirus type 4 antibody. (5) Anti-human IgM. (6) Adenovirus type 4 antigen.

Figure 2. Immunoelectrophoresis showing precipitation of antibody to adenovirus type 4 and immunoglobulins IgG, IgA and IgM. (1) Human anti-adanovirus type 4 antibody. (2) Adenovirus type 4 antigen. (3) Rabbit anti-human IgA, IgM, IgG.

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STUDIES OF ANTIGENIC MATERIALS FROM NEISSERIA MENINGITIDIS*

Ensign Peter W. Soballe
School of Medicine, University of Illinois

Preceptor: R.I. Lytle
Chief, Biochemistry Division

Much of the current research effort at Naval Medical Research Unit No. 4 (NAMRU-4) is directed toward *Neisseria meningitidis*. In the course of these studies, Edwards and Driscoll (1) have devised a fast, easy and reproducible method of extracting a group-specific material which can sensitize sheep erythrocytes for passive hemagglutination (HA) tests. Lytle et al (2) have further purified this material and obtained an acetone-precipitable fraction which shows the same specificity as the original material. This fraction seems to consist principally of carbohydrate, and does not lose HA activity after trypsin, RNase or DNase.

In the present study, analyses were made on *N. meningitidis* whole organisms of groups A, B, C, Boshard (Bo), and group B L-form. Analyses were also made on fractions from the whole organisms, obtained by Edwards' procedure for the extraction of antigenic material, Lytle's acetone-precipitable fraction, and a complement fixing (CF) material from a group B L-form. These analyses included immunologic tests for HA and CF activity, skin tests, chemical determinations for carbohydrate, protein, DNA and lipids, and ultraviolet spectroscopy for DNA and protein.

MATERIALS AND METHODS

Bacteria: *N. meningitidis* organisms of groups A (MKO-1), B (williams B), C (PTS-5) and Bo (isolated from a carrier) were obtained from the Bacteriology Division. The Mycoplasma Research Division furnished lyophilized group B L-form.

Groups A, B, C and Bo were inoculated from frozen sheep blood into Thayer-Martin agar plates, incubated at 37°C for 24 hours, inoculated into 10 ml of Mueller-Hinton broth and re-incubated in

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*From Research Project No. MR 005.09-0086, Bureau of Medicine and Surgery, Navy Department, Washington, D.C.

an incubator-shaker (170-200 rotations/min) at 37°C for 5 hours. They were then inoculated into 250 ml of Mueller-Hinton broth and shaker-incubated at 37°C for 24 hours. Beta-propriolactone (1.5 ml /ml of broth) was added to inactivate the organisms, then refrigerated at 6°C for 48 hours.

Preparation of Fractions (Fig. 1):

Fractions I, II and III: The A, B, C and Bo organisms were collected from the broth by centrifugation (International Centrifuge, 2300 rpm, 1.5 hours, 4°C), washed 3 times in 1.5 M NaCl, and frozen. Since the group B L-form organisms were lyophilized, 200 mg was used. This was considered to be the equivalent of the yield from 250 ml of broth.

Each group of organisms was then suspended in 10 ml of saline, and the pH adjusted to 9.0 with 1N NaOH. The suspensions were incubated for 1 hour at room temperature with constant shaking. They were then adjusted to pH 6.5 with 1N HCl, precipitated with 95% ethanol (5 vol), and centrifuged (2300 rpm, 1 hour, 4°C). The supernatant was separated and marked "Fraction II." The precipitate was resuspended in 50 ml of water and centrifuged (2300 rpm, 1 hour, 4°C). The supernatant fraction, which corresponds to Edwards' antigenic material, was separated and labelled "Fraction I." The residue, designated "Fraction III," was the largest of the fractions. All fractions were dialyzed against running tap water for 48 hours and lyophilized.

Fractions IIIa and IIIb: The separation process was repeated on Fraction III in an attempt to increase the yield of the other fractions.

This fraction was a highly insoluble substance. To remove some of the lipids, 200 mg of the fraction was extracted with 200 ml of a chloroform-ether (2:1) mixture. The residue was labelled "Fraction IIIa," and the extract, "Fraction IIIb." Both were washed 3 times in distilled water and lyophilized.

Fraction IV: Three hundred mg of groups A, B, C, Bo and B L-form were suspended in 20 ml of saline and ruptured in the French Pressure Cell (7000-7500 psi, 3 times). An additional 300 ml of saline was added and the residue removed by centrifugation (2300 rpm, 1 hour, room temperature). The supernatant was separated and treated with phenol (1 vol) in a separatory funnel, and the phenol portion removed. The remaining aqueous portion was

dialyzed against running tap water for 24 hours, and precipitated with cold acetone (3 vol). After centrifugation (2300 rpm, 1 hour, room temperature) the precipitate was collected, lyophilized, and marked "Fraction IV."

L-form CF: The supernatant derived after boiling group B L-form organisms, according to the method of Crawford (3), shows CF activity. This material, marked "L-CF," was donated by the Mycoplasma Research Division.

Analysis:

Immunology: CF and HA titrations were performed on the whole organisms and each of the fractions by the Immunology Division, using the methods described by Edwards (1,4). Group-specific antisera prepared with whole organisms as immunogens were used. One mg/ml concentrations of whole organisms and Fractions I, II and III were used to sensitize erythrocytes. Various concentrations of Fraction IV were used.

Skin tests: As a possible indication of the presence of endotoxin, 1.0 mg of each fraction in 0.1 ml of saline was injected intradermally into 1 of 3 non-immunized male albino rabbits. Skin reactions, judged by size, redness, and general appearance of the wheal, were read after 48 hours and recorded on a scale "0" to "+++." Ten serial dilutions of groups C and Bo materials were made (5.0 mg/ml to 0.009 mg/ml), and 0.1 ml of each dilution injected intradermally. Equivalent dilutions of Dow polystyrene particles (size 1.3 μ) were injected as a control. Skin reactions were read at 48 hours and the highest dilution eliciting a response was noted.

Chemistry: "Total carbohydrates" in the fractions were estimated by the indol method (5) using galactose as a standard. "Total protein" was estimated with the Folin-Ciocalteu phenol reagent (Kabat and Mater p 557-558) using serum albumin as a standard. Total DNA was estimated with the diphenylamine reaction (Kabat and Mayer p 553) using sodium desoxyribonucleate as a standard. A Coleman Universal spectrophotometer was used for the colorimetric analyses and the results were expressed as mg per 100 mg of the dry organisms or fraction. Lipids were identified semi-quantitatively using Oil Red-O (6) and Sudan Black-B (7) on 40 μg of material spotted onto Whatman #3 "chromatography" paper.

Ultraviolet fluorescence: Analysis of the fractions in concentrations of 0.08 to 5.0 mg/ml H_2O, as necessary, to read

within the scale of the Beckman DU spectrophotometer at 2000 to 4800 A, was carried out. Optical densities, recorded at 2600 A° and 2800 A°, were used as indicative of the DNA and protein moieties.

"Purple color": It was discovered that some fractions give a "purple color" when dissolved in concentrated hydrochloric acid and incubated 1-3 hours at room temperature. This color fails to develop in perchloric, hydrobromic, nitric, sulphuric, acetic, or dilute hydrochloric (<2N), or when the solution is heated. This color development does not always occur when the HCl is added to water or saline suspensions of the fractions, even when the generation of heat is carefully avoided; but when 2 ml of concentrated HCl is applied to weighed dry samples (5.0 mg and 50.0 mg), the readings at 6200 A are linear. The readings are also linear when various dilutions of HCl (>2N) are added to constant amounts of material.

RESULTS

Immunology: The results of CF and HA tests on whole organisms and Fractions I, II and III are shown in Table 1. The HA titers obtained using dilutions of Fraction IV are shown in Table 2.

Fraction IV materials (except those from group A) are the only materials showing titers significantly higher than the parent organisms. Edwards' antigenic material, Fraction I, shows only low to moderate titers. Only whole organisms and the "L-CF" antigen-fix complement.

Skin Tests: All materials analyzed evoke skin reactions, as shown in Table 1, while polystyrene controls do not. However, Fraction III appears to contain the greatest concentration of endotoxin. Only 1 µg of Fraction C-III was required to produce a reaction, while all other fractions are considerably less potent than the parent organism.

Chemistry: Results of all chemical determinations are shown in Figure 2.

Carbohydrates make up about 5% of the dry weight of the parent organisms--less (about 1%) for the L-form. Fractions III and IV contain somewhat higher concentrations, while Fractions I and II contain little or undetectable amounts of carbohydrates.

It should be noted that amino sugars are not detectable by the indol method, whereas, gas chromatography** suggested the possibility of substantial amounts of amino sugars in the whole organisms, the chloroform-ether extract of Fraction III, and in Fraction IV.

Protein accounted for about 50% of the dry weight of the parent organisms, and about 60% of Fraction III. Fractions I and II contained only moderate amounts of protein, and Fraction IV contained very little.

DNA was measurable only in the whole organisms and in Fraction III***.

Lipids were present in all of the materials, but tests were only weakly positive for Fractions I and IV.

Ultraviolet Fluorescence: Tracings of Fractions I and III showed broad, high rises in the 260-280 range, but no spikes suggesting high concentrations of a single moiety were detected in any of the materials.

"Purple Color": The "purple color" was detected only in whole organisms, Fractions III and IIIa.

DISCUSSION

Attention is drawn to Fraction IV by its HA-antigenic potency. Less than 2 μg/ml of Bo IV, for example, adequately sensitized erythrocytes. The method of extraction and the chemical analyses suggests that Fraction IV consists principally of carbohydrate and some non-protein impurities.

** Gas chromatography for sugars was carried out on the group C materials, L-form whole organisms, B-IV, and Bo-IV by Abbott Laboratories, North Chicago, Illinois, using a Hewett Packard Model 402 gas chromatograph. There was evidence of mannose and other sugars in all fractions, but peaks possibly representing amino sugars were found only in C-whole organisms, C-IIIb, C-IV, and Bo-IV.

*** In all repetitions of this test, however, even the standards were only slightly colored, 200 μg/ml of sodium desoxyribonucleate having an optical density of only about 20. Lytle et al found as much as 15% DNA in Fraction I (2).

Extracting Fractions I and II from the whole organisms left Fraction III containing a somewhat higher percentage of carbohydrate, protein, and perhaps endotoxin than the parent organism.

This composition of Fraction III, plus the chemical analyses and low antigen titers of Fractions I and II, suggests that these 2 fractions are heterogenous substances containing much protein and no large concentrations of any specific antigens.

Davis et al (8) list the antigens of meningococci as: 1) a somatic nucleoprotein (the P antigen), 2) a somatic carbohydrate, probably a component of the lipopolysaccharide endotoxin, and 3) a group-specific capsular polysaccharide. In 1944, Boor and Miller (9) presented evidence that there is a meningococcus antigen consisting of a polysaccharide-lipid-protein complex, in which group-specificity is contained in the carbohydrate portion.

All of the fractions contain some antigenic materials, but only Fraction IV seems to contain a high concentration. This fraction contains little protein and little endotoxin, and it may, therefore, be suggested that the primary antigen in Fraction IV is the capsular polysaccharide or a "polysaccharide-lipid-protein complex." The group-specificity of Fraction IV demonstrated by Lytle et al (2) certainly tends to support this suggestion.

It may also be suggested that Edwards' antigenic material, Fraction I, with its demonstrated group-specificity, low titers, and chemical compositions, contains small amounts of the same antigenic substance.

With further separation and purification of Fraction IV, this antigenic substance might well be isolated and its structure characterized. The use of a polysaccharide, such as the one we are suggesting here, as a hapten,could, of course, be of great value toward development of a vaccine.

The"purple color" previously described gives a qualitative and a quantitative analysis for some portion of many, but not all, bacteria. This portion is not largely extracted by water, saline, alcohol, or chloroform-ether, since whole organisms treated with all of these (Fraction IIIa) still develop the color while the substances extracted do not. It is, however, completely removed by phenol and/or acetone, since organisms treated with these (Fraction IV) do not develop the color. The chloride ion seems to be necessary, for the color intensity is directly proportional to its concentration, and acids not containing this ion do not seem to elicit the reaction.

Determination of the bacterial substance responsible for the color may result in a useful procedure for a chemical analysis and for classification of bacteria.

TABLE 1. Immunologic Characteristics of Whole Organisms and Fractions I, II and III

	Whole organisms					Fraction I					Fraction II				Fraction III					Fraction IV**		LCF
	A	B	C	Bo	L	A	B	C	Bo	L	A	B	C	Bo	A	B	C	Bo	C_{IIIa}	C	Bo	B
Skin Test*	++	++	++ 8	++ 2	+	+	+	+ 250	+ 16	+	+	+++	+++ 125	+++ 125	+++	+++	+++ 1	+++ 8	+++ 8	+ 31	+ 63	+
CF (1 mg/ml)	1:512	1:256	1:256	1:256	NOT OBTAINED	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-	1.8
HA (1 mg/ml)	1:256	1:64	1:128	1:64	NOT OBTAINED	1:32	1:2	1:8	1:64	1:16	1:4	1:32	1:16	1:16	1:8	1:4	1:16	1:128	1:256	-	-	0

* Includes minimum mg required to produce a reaction for samples which were titered.
No tests performed on LI, CIIIa, A IV, B IV, L IV.

** See Table 2 for HA data.

TABLE 2. HA Titers Obtained With Dilutions of Fraction IV Used to Sensitize RBC's

mg/ml used to sensitize	A	B	C	Bo	B L-form
.002	-	-	-	1:256	-
.005	-	-	-	1:1024	-
.01	-	-	-	1:2048	-
.04	-	-	-	1:4096	-
.1	-	1:64	1:128	-	1:16
.2	-	1:64	1:256	1:1024	1:16
.5	-	1:128	1:256	-	1:32
.1	-	1:256	-	-	-
.2	1.8	-	-	-	-

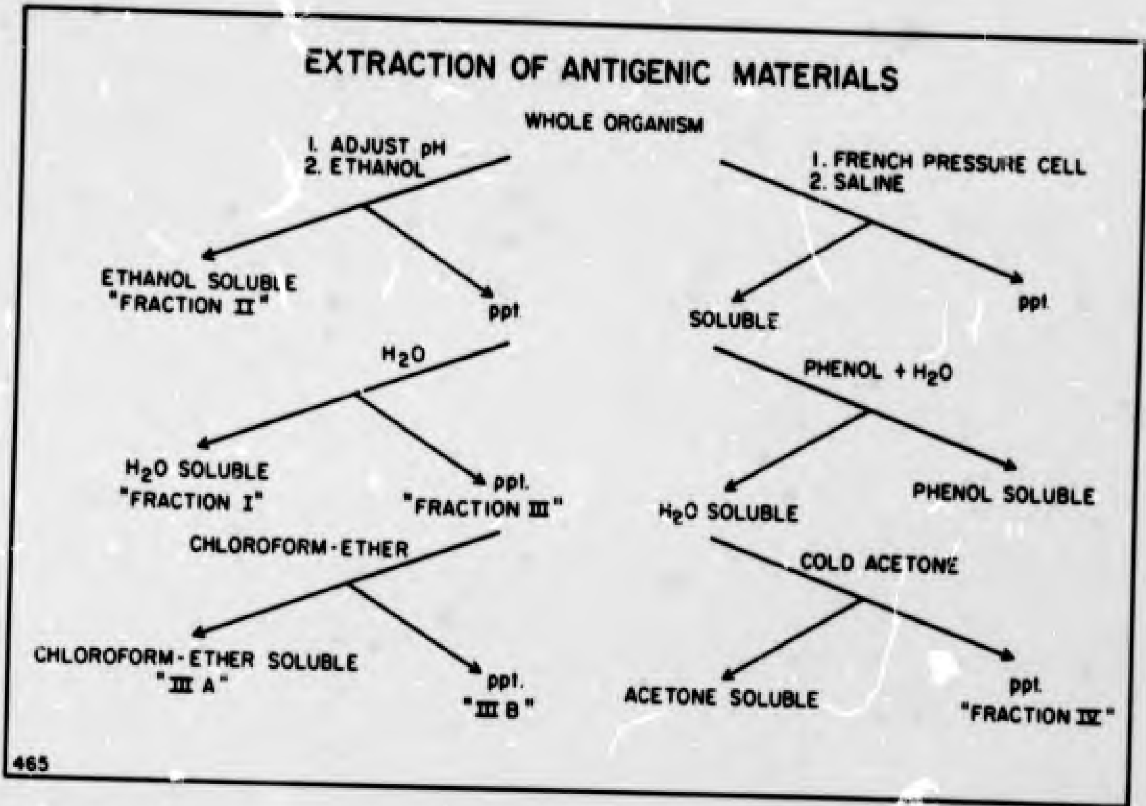

Figure 1

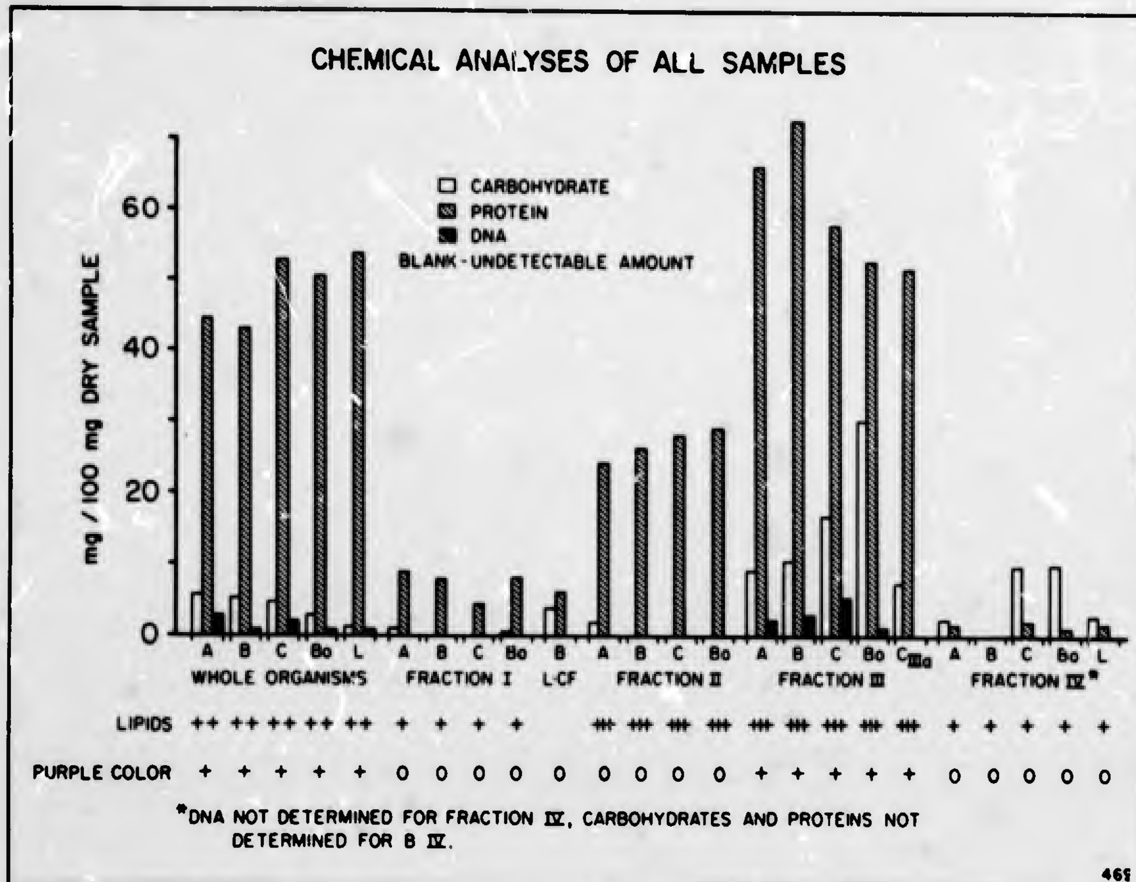

Figure 2

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INCIDENCE OF T-STRAIN MYCOPLASMA IN THE OROPHARYNX*

Ensign Richard T. Swanson
Creighton University School of Medicine

Preceptor: W.H. Kraybill
Chief, Mycoplasma Research Division

The two main areas of the human body which have been studied extensively for the presence of mycoplasma species are the oropharynx and the genito-urinary tracts. However, 1 form of mycoplasma, the T-strain, has not been studied extensively in the oropharynx. Shepard (1) announced in 1954 that he had found a new form of mycoplasma in the urethra of persons with non-gonococcal urethritis (NGU). These he called T-strains, "T" standing for "tiny," since their colonies were much smaller than those of the classical species. Further work has suggested that T-strains might be one of the agents causing NGU (2), while the studies of Ford (3) and Csonka et al (4) have substantiated this work. Isolation of a new T-strain with specific growth requirements from the fetal membranes of a spontaneous abortion has also been reported recently (22).

Mycoplasmas have been known to exist in the oropharynx, oral region, gingiva, and associated areas for many years. It is, in fact, only here that a mycoplasma has definitely been linked to a disease state. Mycoplasma pneumoniae has been found to be associated with primary atypical pneumonia (5). In clinical cases, it is associated most often with the cold agglutinin-reactive type of pneumonia.

Some workers have attempted to use the method of culture developed for the GU system for isolating mycoplasmas from the oropharynx. Ford was able to isolate T-strains from the oropharynx in 5% of a healthy population (3). Serotyping of five such isolates showed them to be similar, serologically, to the genital types. This was done using the metabolic-inhibition test developed by Purcell (6). Hendley et al (7) studied a large population of office workers. Among those of all ages, with acute respiratory disease,

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*From Research Project No. MF 022.03.07-4017, Bureau of Medicine and Surgery, Navy Department, Washington, D.C.

they reported that T-strains were isolated from 2% of 357 pharyngeal samples; however, in the 17-24 age group, the incidence of T-strain mycoplasma was 5.5%.

T-strain mycoplasmas have three distinctive characteristics which aid in their detection. These are their size, their low pH requirement for optimum growth, and their urea metabolism.

Even though Ford (8) has found many other points of similarity between T-strains and large-colony human mycoplasmas, colony size is a useful distinguishing characteristic. Mature T-form colonies rarely exceed 20 microns in diameter (9), however, colonies less than 5 to 6 microns have not been observed. The majority of colonies measured by various workers have been in the range of 14 to 24 microns (10). In contrast, colonies of M. hominis are generally 200 microns in diameter.

The typical morphological appearance of T-strain colonies, when observed under low power (100x), is that of minute irregular colonies devoid of surface growth, according to Shepard (9). Ford and Macdonald, however, have published photographs which have been interpreted as showing surface growth (8). Living unstained T-strains, when examined through the bottom of the culture plate, exhibit a refractile appearance in reflected light. Differentiation from pycnotic and deposited cellular material can be made by the heavier density of the microorganism.

Because of their small size, gross microscopic identification is difficult, especially in clinical material where crowding has stunted growth of large, classical PPLO (9). The best method of identification is by the wet, stained-block technique of Dienes (9,11). In a later publication, Shepard claims that this method will allow differentiation based on the color of the stained colonies (10). T-strains are said to show a greenish-blue coloration.

The second area in which the T-strain colonies differ is the pH at which they show optimal growth. When mycoplasmas were originally isolated from human material, it was found that a pH of 7.8 was optimal for their growth. Thus, this value was used in the original studies of T-strains, but extreme difficulty was encountered in achieving growth on subculturing. Ford investigated this by growing stock cultures at pH 7.0, 7.4 and 7.8 in 10% CO_2, and found little difference in them (12). He then continued to use pH 7.8 conditions. Shepard, however, investigated this and found pH 6.0 to give larger colony size and better viability in both broth and agar cultures.

This modification was found to alleviate the problem of propagation beyond primary isolation (13).

Kraybill and Crawford (14) have investigated pH variation in growth of mycoplasma on plates from the human oropharynx of field isolates and found that, excepting M. pneumoniae, the human oral mycoplasmas are more frequently isolated at pH 6.0 and 7.0.

The rapid loss of viability in broth after 16 to 18 hours led to the search for a toxic substance which was a growth product. None was found. Instead, attention was turned to the composition of the media to see if a needed metabolite was being depleted. Horse serum, the usual supplement, was examined and found to have a urea concentration of 40.0 mg/100 ml (15). Serum treated with a commercial urease was rendered completely inactive as a supplement (15). Thus, T-strain mycoplasmas were found to need urea for proper growth and division.

Ford found that incorporation of 0.05% urea into routine media resulted in more uniform growth of T-strains to a higher titer (20×10^6 to 30×10^6 colony-forming units (CFU)), than unsupplemented broth (16). Coupling this with the addition of 0.002% phenol red gave a readily observable indicator of growth, as the pH rose due to the metabolism of urea to ammonia, changing the indicator from yellow to red.

The initial concentration of urea in the system is also important. Normal untreated horse serum has a urea concentration of 40.0 mg/100 ml. Normal media used have a concentration of about 0.003% urea. It has been found that a low initial concentration of 0.01M (0.06%) of urea produced maximal growth and urease activity of T-strains. Shepard has found no difference in titer between media with 1.0% and 0.5% urea after 48 hours incubation, but urease activity was highest at the lower concentration (15).

In addition, the effect of pH has been studied and it has been noted that urease activity is highest at pH 6.0, thus, explaining the maximal growth of the organism at that level (15).

Biochemically, T-strains can be distinguished from classic human mycoplasma since T-strains are inhibited by 1:500 thallium acetate in primary cultures. This antibacterial agent is commonly added to media to inhibit gram-negative organisms. However, it has been observed that stock purified T-strains, which have been propagated in the laboratory, gradually develop resistance to the inhibitory properties of thallium acetate (10). Second, T-strain mycoplasmas are selectively inhibited by erythromycin *in vitro* (17). With the exception of *M. pneumoniae*, all human classic PPLO strains tested are resistant to erythromycin. This has been used as a test to indicate the presence of T-strains in NGU. The presence of

6.25 μg/ml of this antibiotic in pH 7.5 culture media completely inhibited the growth of T-strains, which were concurrently noted on identical plates without erythromycin (17). There is disagreement, however, on the dosage of erythromycin needed to inhibit growth. Taylor-Robinson reports that 64 μg/ml was needed to inhibit the characteristic phenol-red color change when incubated in microtiter test wells (18); whereas, Shepard reports that only 0.78 μg/ml is needed at pH 7.5 and 3.12 μg/ml at pH 6.0 when incorporated into his media. The difference in dosage at the latter of the two pH values is due to the inhibitory effect of the lower pH on the erythromycin (17).

In this investigation, the growth potentials of several media were examined using a stock culture, and the best one was used to isolate and subcultivate T-strains from the oropharynges of Navy recruits. Carrier rates were compared with those obtained for civilian populations, and an attempt was made to identify differences in T-strain levels between recruits with and without acute respiratory conditions. The possible relationship between acute respiratory disease and the presence of T-strain mycoplasma was examined.

MATERIALS AND METHODS

Three stock cultures of T-strains, obtained from several sources, were used in testing various media. They were: the Boston T-strain, T-24, and T-960**. The Boston strain was isolated from the fetal membranes of a spontaneous abortion while the other two were isolated from the urethral tract.

Initially, the growth potentials of two T-strain media were investigated. These were a standard PPLO media without thallium acetate (12) and a special trypticase soy media, designated A2 by Shepard (10). Also used was a broth modification of the A2 media, deleting only the Ionagar No. 2.

The agar media have been described by Shepard (10) and the only modification made in this study was that the pH of the horse serum was lowered to pH 6.0, then the penicillin was added prior to adding it to the basal media. This differs in that Shepard had calculated how much sterile acid was needed to bring the 40 ml of serum to the proper level, and added this to the basal media

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**The Boston T-strain was kindly supplied by Dr. Ruth Knudsin of Peter Brent Brigham Hospital, Boston, Mass.; the T-24 strain by Dr. Denys Ford, Vancouver General Hospital, Vancouver, BC; the T-960 strain by Dr. Maurice C. Shepard, Naval Medical Field Lab., Camp Lajeune, N.C.

before adding the unadjusted serum. However, the present method seemed to cause no difficulties, and no precipitation or cloudiness was observed in the horse serum.

Shepard had used the phenol red and urea supplement only in broth culture, but here it was incorporated into agar media following Williams and Taylor-Robinson (19).

Because of the paucity of initial positive results in comparing culture media, only the A2 media was used in clinical sampling. Those primary isolation plates, which appeared to be positive for T-strains after incubation, were block subcultured to other plates of A2 agar media to which 1% phenol red (0.05% solution) and 1% wt/vol crystalline, Merck pure urea had been added.

Recruits at the Great Lakes Naval Training Center who reported to sick call were separated into 2 groups, according to their complaint. These who had respiratory complaints (ARD) were designated the test group and those whose complaints were non-respiratory and who had no respiratory symptoms were designated the control group. Fifty men from each group were sampled by passing a long-stemmed cotton swab over the oropharynx, and inoculating these immediately onto plates of A2 agar without phenol red. These plates were incubated anaerobically for 48 hours at 37° under 5% CO_2-95% N_2, using 3 cycles of evacuating and replacement with the gas mixture. All of the controls were incubated separately from the test group.

Upon completion of incubation, the plates were read under the low power of a binocular dissecting microscope. Tabulation was made of those plates having large colony growth and suspected T-strain growth. Determination of T-strain growth was done by gross morphological size and shape only.

Plates showing possible T-strain growth were subcultured by the push-block method on A2 agar plates containing phenol red and urea. These were incubated anaerobically for 48 hours at 37° and examined for characteristic color change.

On all of the positive plates, ammonia could be detected either by smell or by volatilization by sterile 1N NaOH. Red litmus paper was placed over these and a blue color change was taken as an indication of a volatile basic substance.

Erythromycin sensitivity was ascertained with subcultured colonies on A2 media using commercial erythromycin discs (Baltimore Biological Laboratories Inc.) containing 2 or 15 µg/disc.

RESULTS

Table 1 gives the results of the investigation of several media. Using all three stock cultures on each, only the strain T-960 proved viable and showed good growth. The T-24 strain showed some growth indicated by a color change in A2 phenol red broth after 24 hours of aerobic incubation, but growth could not be stimulated on agar or in further subculture. The Boston T-strain failed to grow on any of the media used. In all subsequent work the A2 media, both broth and agar, was used. Urea, used in a concentration of 1.003%, proved satisfactory.

The plates taken from the recruit population showed 36 (72%) of the test group and 26 (52%) of the control group with large colony growth (Fig. 1). Twenty-seven (54%) of the test group showed possible T-strain growth and 18 (36%) of the control group showed the same. Only 2 of the test group which showed T-strain growth did not show large colony growth.

Positive plates were subcultured onto A2 agar plates with phenol red and 7 (14%) of the test group showed a positive color test. Two (4%) of the controls did the same. These 7 test plates and 2 control plates were subcultured again and an antibiotic disc of 2 µg/disc placed on them. After 48 hours anaerobic incubation, 7 of the test group subcultures and 1 of the control group showed a color change. On all of the plates, growth of T-form and of large colony form could be detected, but no rings of erythromycin inhibition could be observed with the 2 µg/discs. A further subculture was done, and a disc of higher concentration (15 µg/disc) was used. Again, on all plates, there was no ring of inhibition, but on 6 test plates there was heavy growth of T-strain-like organisms in a tight ring around the disc with very little growth elsewhere.

DISCUSSION

Several interesting facts were noted. One concerns the length of incubation time. As reported in the literature, 48 hours is the maximum time before viability begins to wane. However, it was noted in this study that microscopic visualization of the organisms was difficult or impossible after this period of incubation. It was found that sealing the plate with normal sealing wax and allowing it to remain at room temperature for an additional 24 hours increased size differential of the organisms without loss of viability. Viability was demonstrated by subculture onto agar and into broth with subsequent growth. It was also found that the A2 agar media has a high water content and was highly susceptible to fungal and bacterial contamination, despite the 0.1 ml of Amphotericin B (5 mg/ml) that was used per 100 ml of final media.

The large colony growth on the plate was extraordinary in that it grew to large size in only 48 hours, and a pH 6.0 medium does not appear to inhibit large colony growth. This is in contrast to the longer period of growth usually required for large colony growth. Species determination of this large colony population was difficult; however, a typical colony picked from each of 4 plates was identified as M. hominis from 3 plates and M. salivarium from the fourth. Perhaps the use of these media have modified a known strain in some way, speeding growth and leading to a change, which temporarily resists classification by known antisera. Further investigation into the growth potential of this agar for large colony growth should be pursued.

A trend may be seen towards a higher percentage of recovery of T-strains in recruits with acute respiratory complaints compared with those ill with non-respiratory complaints. However, due to the small number of samples taken, no statistical significance can be attached to the findings. It would appear, however, that there is an increase in the incidence of T-strains during acute respiratory disease, although, no definite etiological relationship can be inferred from this fact. This 14% incidence of T-strains found in ill recruits is also higher than that found by Hendley et al (7) who reported a 5.6% incidence in the 14-24 age group of a large civilian population ill with acute respiratory disease. No reason can be found for the low percentage of large colony recovery of the control population. Other studies (21) have shown a much higher percentage of recovery. Comparison of the percentage of T-strain isolations in the control group with that reported in the literature is favorable. Ford reported 5% of a healthy population (3). Thus, it might be suggested that the 4% figure for a healthy recruit population differs very little from the 5% in a civilian population.

SUMMARY

An attempt was made to determine the prevalence of T-strain mycoplasma from the oropharynx of a recruit population at the Great Lakes Naval Training Center, and to investigate whether these were to be found in greater numbers in recruits with ARD compared with those with non-ARD complaints. Greater numbers were isolated from those with ARD complaints (7 vs 2) as confirmed by morphological and biochemical means, but no statistical significance can be drawn. It was also shown that the A2 media developed by Shepard (10) proved to be excellent for the growth of T-strain isolates. Further, it was shown that large colony mycoplasma of undetermined identity would grow to large size in 48 hours, under the conditions of low pH.

TABLE 1. Results of Media Study

Stock Culture	Media	Mode of Incubation	Growth on Primary Culture	Growth on Subculture
	PPLO	Anaerobic	Negative	Negative
T-24	A2 agar	Anaerobic	Negative	Negative
	A2 broth	Anaerobic	Positive	Positive
	PPLO	Anaerobic	Negative	Negative
Boston T	A2 agar	Anaerobic	Negative	Negative
	A2 broth	Anaerobic	Negative	Negative
	PPLO	Anaerobic	Not Investigated	
T-960	A2 agar	Anaerobic	Positive	Positive
	A2 broth	Aerobic	Positive	Positive

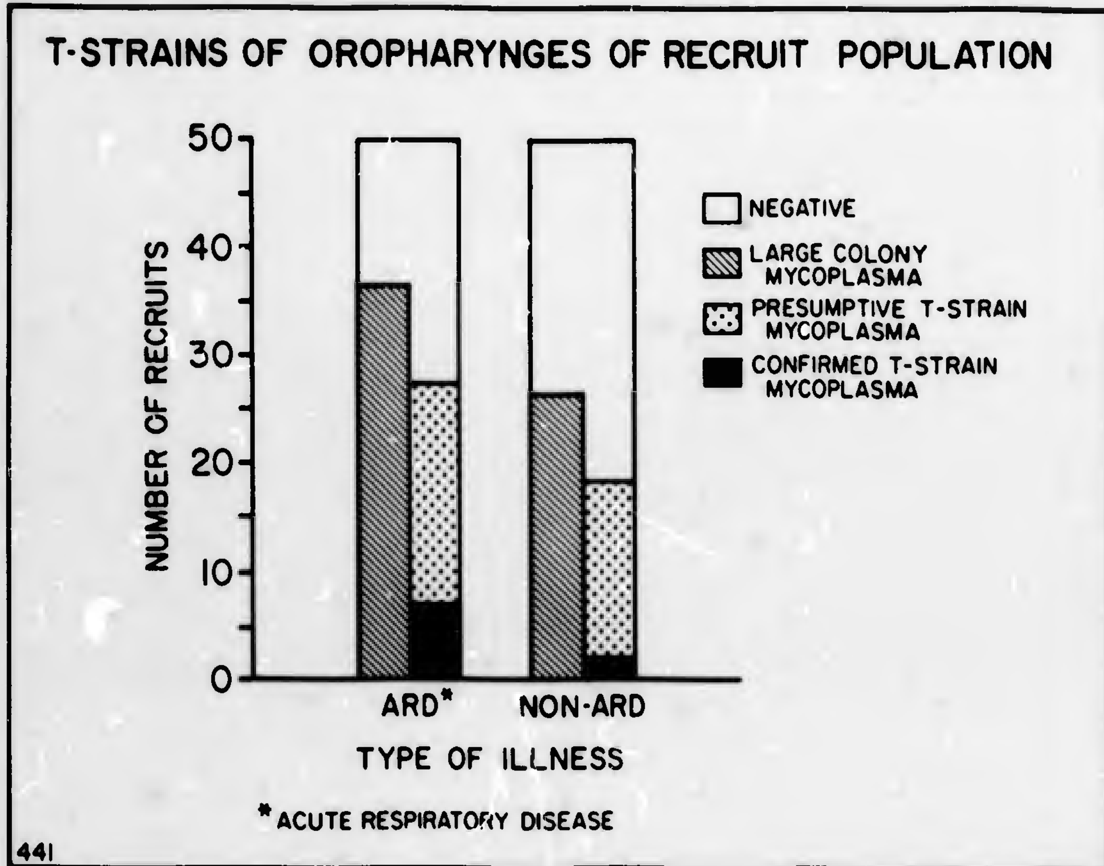

Figure 1

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13. ABSTRACT

This report contains the final reports of the research projects done by the research clerks assigned to the Naval Medical Research Unit No. 4 during the summer of 1967. The titles of the projects are as follows:

1. The Enhancement of Mouse Virulence and Immunologic Cross-Protection of *Neisseria Meningitidis*.
2. The Association of Organ Antibodies with Pneumonia and Acute Respiratory Disease in Navy Recruits.
3. Preliminary Report on Interferon-Like Responses of Naval Recruits Following Yellow Fever and Other Immunizations.
4. New Media for Isolation of Mycoplasma.
5. Immunoglobulin Specificity of Adenovirus Type 4 Antigen.
6. Studies of Antigenic Materials from *Neisseria meningitidis*.
7. Incidence of T-Strain Mycoplasma in the Oropharynx.

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14. KEY WORDS	LINK A		LINK B		LINK C	
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5. Adenovirus; antibody						
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