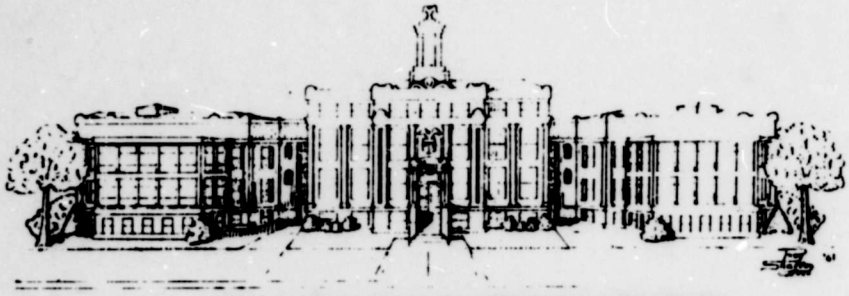
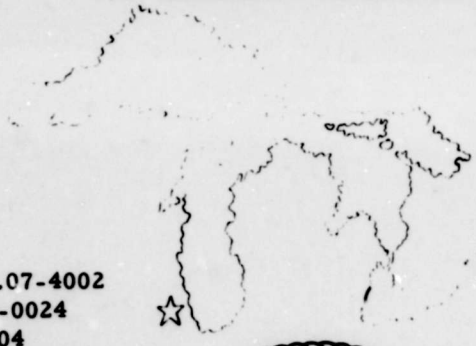
NAVAL MEDICAL RESEARCH

UNIT No. 4



1966 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) experience at Naval Medical Research Unit No. 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 Naval Medical Research Unit No. 4 is to conduct research on the epidemiology, etiology, and methods of prevention and control of acute communicable diseases of the respiratory tract.

Naval Medical Research Unit No. 4 offers a unique opportunity to the research clerks in offering a choice of field trials, laboratory study, or 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 viral diseases.

The unit offers its resources to the clerks in the process of conducting their investigations. These 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
Officer in Charge

PREFACE

This report consists of scientific papers which are the consummations of research work done by the research clerks assigned to the Naval Medical Research Unit No. 4 during the summer of 1966. 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.

Close supervision and direction were given the clerks during the initial phase to ensure their knowledge of the problem, objectives desired, and research and laboratory techniques to accomplish the study. The clerks pursued the studies to completion, involving (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 final report. In keeping with the philosophy of the clerk experiencing all phases of the study, the enclosed report reflects individual efforts. Each paper was reviewed by the respective preceptors, but changes were kept to a minimum.

The individual studies were complemented by a formal lecture series given by members of the Scientific Department so that all clerks were exposed to the underlying concepts of microbiology, immunology, biochemistry, biometrics and allied fields. The scientific staff of NAMRU-4 feels this program is well worthwhile. The techniques and skills needed to accomplish applied research, along with its frustrations, can only be a valuable and broadening experience to each research clerk who has participated in this program.

D. S. HOWARD
CDR MC USN
Editor
1966 Research Clerks Reports

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THE EFFECTS OF GROUPING ON ANTIBODY PRODUCTION AND DEATH RATES AMONG MICE

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Current studies indicate that conditions of prolonged stress will induce enlargement of the adrenal glands (1, 2, 4). As a consequence of this hypertrophy, the adrenocortical steroid output of stressed animals is measurably increased (3, 6). This increased steroid level may inhibit the antibody response of an organism (7). At this time, a number of problems remain in this area; for example, the specific pathways of antibody production suppression and the occasionally paradoxical relation between antibody titer and disease resistance remain to be solved.

Working with the laboratory mouse, Vessey (7) found that grouped mice have significantly lower titers of circulating antibody than do isolated mice. Dominant mice were also found to have higher titers than others amont their group.

According to Christian (4), grouping mice in sufficiently crowded populations was a suitable stress for the inducement of adrenal hypertrophy. This hypertrophy was not directly proportional to population density, but exhibited an optimum at some specific grouping size. This size was dependent on the social-rank society existing in mouse colonies and was determined by both the frequency of contact and the freedom with which one mouse may pursue another.

The plan of this study was to repeat portions of the work cited above and to provide a pilot study of some of the problems outlined. Hence, the antibody titer of immunized mice subjected to various degrees of stress due to crowding was measured. Subsequently, the populations were challenged with lethal doses of a suitable bacteria and the death rates determined.

MATERIALS AND METHODS

Initially, 500 female albino mice (Swiss Webster strain), weighing

17-19 grams, were separated into groups of 5 mice each for purposes of stabilization. Each group of 5 was housed in a standard 11 x 7" plastic cage. Physical limitations prevented the optimal isolation of individual mice. After one week, all mice were injected with .5 ml of typhoid-paratyphoid vaccine and grouped into populations as indicated in Table 1. Each population was again housed in an 11 x 7" cage for the remainder of the experiment. After 9 days, approximately .4 ml of blood was extracted from the orbit of each mouse. This blood was centrifuged in a Beckman Microfuge for 5 minutes and one drop of serum was obtained for antibody titer determination by the bacterial agglutination method. Titer determinations were run on half of the mice from each population resulting in a 50% sampling (see Table 1). The agglutination tests were conducted in 10 x 70 mm test tubes using Salmonella O antigen, group B (Difco Lab), as the test antigen. The initial serum dilution was 1:10 in saline followed by 5 two-fold dilutions in successive volumes of .2 ml, terminating in a 1:320 dilution. Two-tenths of the appropriate antigen was added drop-wise to each serum dilution after which the tubes were incubated at 37°C for 1 hour and subsequently refrigerated at 18°C overnight. The total volume of antigen, plus diluted serum, was .4 ml in each tube. Before reading, the tubes were centrifuged at 1500 rpm for 10 minutes to make observation of the bacterial agglutination easier. The tubes were read and the endpoint was considered to be the highest dilution of mouse serum giving a 1+ agglutination.

The day following bleeding, all the immunized mice and 10 non-immunized controls were injected with 1 ml of a 1000 LD_{50} dose of Salmonella typhimurium. This dosage was a 1:100 dilution of an 18-hour bacterial culture grown in brain heart infusion broth at 37°C. The LD_{50} dose was previously determined by injecting several groups of non-immunized mice with various dilutions of an 18-hour culture of Salmonella typhimurium. The 1000 LD_{50} was pre-determined by injecting several groups of immunized mice with varying LD_{50} doses.

After challenge with the 1000 LD_{50} of S. typhimurium, the grouped mice were observed for 8 days and the death rates determined for each population.

RESULTS

Previous work had indicated that the LD_{50} for non-immunized mice was a 10^{-5} dilution of an 18-hour culture of S. typhimurium. This was

confirmed and the LD_{50} was achieved by the fourth day following injection of this dosage. The results of the LD_{50} determination for immunized mice are shown in Table 2. A 1000 LD_{50} or a 1:100 dilution was chosen from these results.

Initially, 40 mice per cage was chosen to be the most dense population. However, within 2 to 3 hours following this grouping, 75% mortality occurred, while no deaths were observed in other groups containing fewer mice. Because of this, the maximum was reduced to 30 mice per cage after which no deaths were recorded.

The results of the antibody titer determinations are shown in Figures 1, 2, 3 and Table 1. From these, it can be observed that many mice apparently had no detectable antibody titer. However, a larger percentage of mice in the smaller populations exhibited a titer than did those in the larger populations (Fig. 3). Of those mice which did respond, the average titer was remarkably constant in all populations (Fig. 1). However, if those mice in each population, which did not respond, are included, the smaller populations have a higher average antibody titer (Fig. 2).

From the death rates (Fig. 4), it can be seen that the populations with 2 mice had the highest mortality. Those populations containing more animals apparently survived more successfully. The 10 non-immunized control mice were dead by the fourth day following injection.

DISCUSSION

The results of this experiment differ significantly from those of other workers (7) in that the percent of animals responding to immunization is the parameter which is most profoundly affected by grouping rather than the mean antibody titer. Perhaps this discrepancy is due to the different antibody stimulating vaccine used in this experiment or to the difference in titer determination methods.

The reason for the remarkable death rate in the mice grouped in two's is not known, especially when over 50% of them had responded with measurable antibody. Perhaps the greater degree of movement or higher temperatures in the denser populations is related to this.

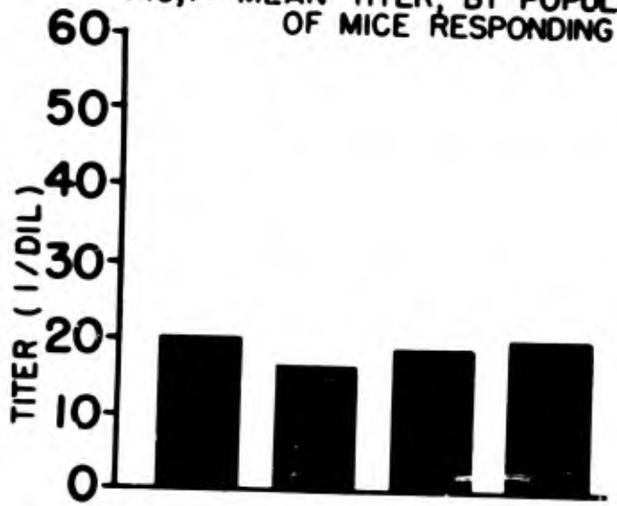
TABLE 1 -- Summary of Experiment

Number of mice per population	Number of populations	Total number of mice per population	Number of mice tested for antibody	Percent of population responding	Mean titer of those responding	Mean titer of entire population	Death rate as percent of population
30	3	90	45	20	20	4	13
20	6	120	60	22	18	4	6.6
4	10	40	20	50	16	8	7.5
2	20	40	20	55	20	11	35

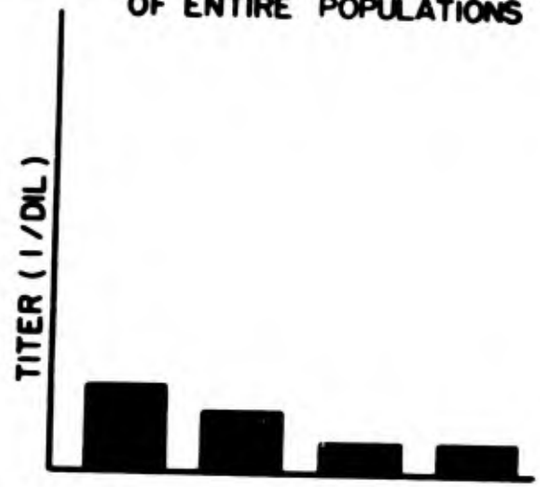
TABLE 2 -- Number of Mice Dying out of Groups of 10 "Immune" Mice

LD_{50}	Days after injection death occurs									
	1	2	3	4	5	6	7	8	9	10
Dose: 5	0	0	0	0	0	0	0	0	1	0
50	0	0	0	0	0	0	2	0	1	0
1000	0	0	0	0	3	1	0	0	0	0
Undilut.	6	3	0	0	0	1	-	-	-	-

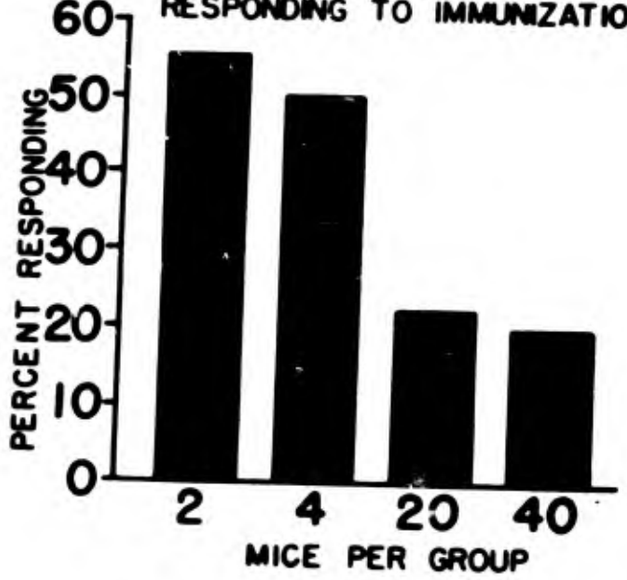
FIG, 1: MEAN TITER, BY POPULATION OF MICE RESPONDING



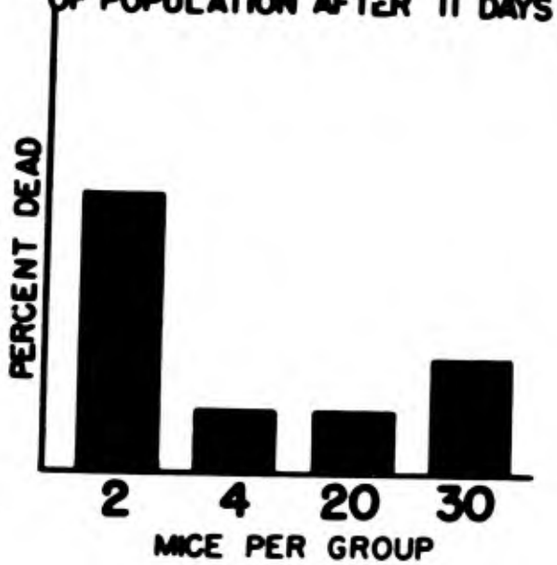
FIG, 2: MEAN TITER, BY POPULATION OF ENTIRE POPULATIONS



FIG, 3: PERCENT OF POPULATION RESPONDING TO IMMUNIZATION



FIG, 4: DEATH RATES AS PERCENTAGE OF POPULATION AFTER 11 DAYS



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THE EFFECT OF PENICILLIN ON THE ORAL MYCOPLASMA FLORA OF NAVAL RECRUITS

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Preceptors: York E. Crawford
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and

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In the winter of 1961-62, a study was made of mycoplasma in the throats of naval recruits undergoing penicillin prophylaxis. Crawford (1) noted a tendency for negative individuals to convert to a positive status when under the influence of penicillin. It is known that penicillin can induce bacteria to grow into the L, or Lister form, in vitro. Since L forms resemble mycoplasmas in appearance, it seemed reasonable to assume that penicillin administered in vivo could cause an increase of mycoplasmas in the human oropharynx.

Subsequent investigations carried out by Knudson (2) and Lyons (3) followed up this hypothesis with studies on the recruit population. Incubation time, insufficient numbers of positive cultures, or the inadequacies of existing laboratory methods hampered these investigations.

This study was undertaken with similar objectives, but with the aid of a more exact mycoplasma identification technic. However, the validity of results was improved by decreasing the number of specimens and making more detailed identifications of the mycoplasma flora of each subject.

MATERIALS AND METHODS

The population consisted of naval recruits at the Great Lakes Naval Training Center in the July-August period of 1966. At the end of the second week of training, 1.2 million units of benzathine penicillin G

were administered intramuscularly in the gluteal region. Approximately 5% of these recruits were allergic to penicillin and did not receive the injection. The benzathine penicillin used is a long-lasting drug which remains in the region injected as a solid mass which slowly dissolves. This provides prophylaxis against streptococcal infection by maintaining low levels of penicillin in the blood stream from 4 to 6 weeks. As a protective measure for the allergics they were cultured by throat swab to detect the streptococcus, if pres

In this investigation, those receiving penicillin comprised the experimental group and those who were allergic constituted the control group. The sample consisted of 20 men in each group. The man who claimed to be allergic to penicillin and the man next in line who received the prophylaxis were considered an experimental pair.

The throat of each man was swabbed bilaterally in the tonsillar region. A methylene blue plate (4) was inoculated with this material for the isolation of Mycoplasma pneumoniae. Also, a plate containing the PPLO agar medium (5) of Shklair, et al, was similarly inoculated for the isolation of other mycoplasma species. The methylene blue plate was sealed with paraffin and incubated at 37°C for 30 days. It was examined weekly and declared negative only after 30 days of incubation.

The second plate was incubated in an atmosphere of 95% N_2, 5% CO_2 (5). After two weeks at 37°C, it was declared negative if no growth had appeared. If the plate was positive, 10 colonies were selected at random, and each of these colonies was transferred to a fresh plate which was again incubated under the same conditions for a period of 10 days. Positive plates were examined, and this time a single colony was re-cultured on fresh media. This procedure was carried out once again, for a total of 3 cloning steps. All plates that became negative during the subculture procedure were re-cultured from the original sample in order to obtain a full complement of 10 colonies from each primary plate.

The object of these cloning steps was to obtain a reasonably pure culture of a single species. At this point, a 1-cm square block of agar was cut from an area containing approximately 15 colonies. This was dropped into a vial containing 3 ml of saline, which was then vigorously shaken using a vortex mixing machine. Then .3 ml of the saline suspended organisms was transferred onto a plate of Shklair's medium. This plate was allowed to dry in a 37°C incubator for 15-20 minutes. A multidisk containing specific antisera (6, 8) was next placed on top of the agar. After one week of incubation, under anaerobic conditions, the plate was examined

for zones of growth inhibition and the species was recorded.

Any M. pneumoniae recorded by this technique were subjected to the tetrazolium color test (4).

At the end of 10 days, the same recruits were again assembled and a second throat culture was taken. These plates were treated in the same manner as described above.

RESULTS

Of the 40 recruits in the sample, none of the cultures were positive for M. pneumoniae, either before or after penicillin administration. This was expected because of the small sample involved.

Data from species determinations of isolates, made on the anaerobic plates, were analyzed in three perspectives:

First, as a general assessment of growth, Table 1 shows the distribution of recruits whose throat swab material contained mycoplasma before and after the penicillin administration. It is apparent that both experimental and control groups showed similar tendencies.

Second, the change in the distribution of positive cultures before and after penicillin administration was analyzed. Three categories were possible: (a) a positive carrier reverting to a negative status: (b) a negative carrier converting to a positive carrier, or (c) either a positive or a negative carrier remaining the same. Table 2 illustrates that in both cases, the tendency here was for a positive carrier to revert to negative status.

Third, the incidence of mycoplasma species and mixed flora in positive plates before and after penicillin administration was analyzed, and the results are shown in Table 3. These figures are expressed as the percentage of positive plates. In other words, in the first culture of the control group, all of the positive plates contained at least one M. salivarium colony. The figures do not represent the percent of a species on a positive plate. This can only be obtained by subculturing all of the colonies on a positive plate. This analysis shows that positive plates from both the experimental and control group contained a similar distribution of mycoplasma species. This distribution remained constant in both groups after administration of penicillin.

Three different mycoplasma species were isolated in this study; M. salivarium was most dominant, followed by M. pharyngis, with M. hominis found in only one case.

SUMMARY

Although, at some time during this experiment, there occurred a drastic reduction in mycoplasma flora, the penicillin prophylaxis was not the causative factor. These results indicate that neither gains nor losses of mycoplasma, nor individual species of mycoplasma, were induced by the administration of penicillin.

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TABLE 1. -- Recovery of Mycoplasma from the Oropharynx of Recruits before and after Penicillin Prophylaxis, Great Lakes, Illinois, July-Aug 1966

Culture period relative to penicillin prophylaxis	Test group	Mycoplasma Cultures		
		Positive	Negative	Total
Before	Experimental	13	7	20
	Control	15	5	20
	Subtotal	28	12	40
After	Experimental	5	15	20
	Control	3	17	20
	Subtotal	8	32	40
	Grand total	36	44	80

TABLE 2. -- Change in the Distribution of Positive Mycoplasma Cultures Obtained from the Oropharynx of Naval Recruits as a Result of Penicillin Administration, Great Lakes, Illinois, July-Aug 1966

Change in Mycoplasma culture status	Test Group		
	Experimental	Control	Total
Positive to negative	10	12	22
Negative to positive	2	0	2
Unchanged	8	8	16
Total	20	20	40

TABLE 3. -- Mycoplasma Species Present in Positive Cultures from the Oropharynx of Naval Recruits before and after Penicillin Administration Great Lakes, Illinois, July-Aug 1966

Test group	Time of culture relative to penicillin	Distribution of Mycoplasma Species in Positive Plates			
		M. Saliv.	M. Phar.	M. Homi.	M. Sal. + M. Phar.
Experimental	Before	11/13*	4/13	0/13	3/13
	After	5/5	2/5	0/5	2/5
Control	Before	15/15	6/15	1/15	6/15
	After	3/8	1/3	0/3	1/3

*Numerator = Number of plates with indicated species.

Denominator = Total number plates positive.

STUDIES ON THE MECHANISMS OF SULFADIAZINE RESISTANCE AMONG MENINGOCOCCI

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Some isolates of Neisseria meningitidis have been found to be resistant to sulfadiazine (1). The reason for sulfadiazine resistance in the meningococcus has not been established conclusively. Evidence indicates that this resistance is achieved by an increase in production of paramino-benzoic acid (PABA), which alters the competitive inhibition by sulfadiazine (2).

Recently, Brooks (3), in his studies of this subject, found that Daraprim (pyrimethamine compound), although not inhibitory itself in concentrations below 100 μg/ml, did make sulfadiazine-resistant meningococci susceptible to sulfadiazine in vitro.

Several antibiotics have been found to which sulfadiazine-resistant organisms are sensitive; although, none has proved effective for mass control of the carrier state, which is of importance in a military installation (4).

The purpose of this study was to establish: 1) whether or not the PABA-folic acid complex is concerned with sulfa-resistance of the meningococcus; 2) if it is, at what point in the metabolic pathway does resistance develop; and 3) what drugs can be used to block or alter such metabolic pathways.

MATERIALS AND METHODS

The strains of meningococci used in these experiments were chosen from among strains isolated from the nasopharynx of military personnel undergoing training at the Naval Training Center, Great Lakes, Illinois. Cultures were obtained as part of a surveillance program in the detection and control of meningococcal infections. Sulfadiazine sensitivity determinations of the strains were conducted by personnel of the Bacteriology

Division, Naval Medical Research Unit No. 4, in the course of their routine work employing the method described by Frank, et al (5).

Phase I -- Establishment of Pathways of Resistance.

In this phase of the investigation, compounds which are dependent on or involved in folinic acid synthesis (that is, paramino-benzoic acid, folic acid, adenine sulfate, and DL-serine) were incorporated into a medium to observe the antagonistic effect on sulfadiazine sensitivity of resistant and sensitive strains of meningococci. Strains representing various levels of sulfadiazine sensitivity varied from 20 mg/100 to 0.05 mg/100.

A checker-board arrangement was devised by adding various concentrations of sulfadiazine to various concentrations of the antagonist. Sulfadiazine concentrations ranged from 0.05 mg/100 to 100 mg/100. A molar solution of antagonist was prepared which was equivalent to 20 mg/100 solution of sulfadiazine. The following dilutions of the antagonist were then made: Undiluted, 1 to 10, 1 to 100, 1 to 500, and 1 to 1,000. Dilutions of antagonist and sulfadiazine solutions in the amount of 0.15 ml were added to approximately 15 ml of Mueller-Hinton medium.

Phase 2 -- Alteration of Resistance.

Daraprim (2, 4-diamino-5-para-chloro phenyl-6-ethyl pyrimidine), which can alter or block pathways for the formation of formyl groups, and isonicotinyl hydrazide (isoniazid), which combines with DNA and RNA and alters metabolic pathways, were tested for any potentiation of inhibition by sulfadiazine. In one phase of the potentiation experiment, solutions of Daraprim and isonicotinyl hydrazide in equal molar concentrations to the antagonist of the first part of the experiments, were incorporated in a checker-board manner. In another phase, various 2-fold dilutions of only Daraprim ranging from 100 μg/ml to 1.5 μg/ml were added to agar containing various concentrations of sulfadiazine. A set of plates containing the various concentrations of sulfadiazine only were also prepared. The sensitivity of strains under study to sulfadiazine alone was then compared to that in the presence of various concentrations of the potentiators.

RESULTS

Phase 1.

Only PABA altered the inhibitory effect of sulfadiazine (Table 1).

Adenine sulfate, DL serine, and folic acid showed no activity at the concentrations used in this experiment. Table 2 shows the effect of PABA on sulfadiazine activity. As can be seen, smaller amounts of PABA are needed to alter the sulfadiazine sensitivity of resistance strains. In Table 3 are shown the same data expressed as molar ratios of sulfadiazine to PABA. As can be seen with the sulfadiazine-sensitive strain, from 5 to 75 moles of sulfadiazine are inactivated by each mole of paramino-benzoic acid. On the other hand, with the sulfadiazine-resistance strains, from 50 to 2,023 moles are inactivated per mole of paramino-benzoic acid.

Phase 2.

The first few experiments with the pontentiator drugs Daraprim and isonicotinyl hydrazide were unsuccessful. However, when Daraprim was employed at concentrations of 1.5 μg/ml to 100 μg/ml there resulted an alteration in sensitivity from 3 to 15 times, depending on the original sensitivity of the organism (Table 4). It will be noted that strains classified as sulfadiazine-sensitive became from 3 to 6 times more sensitive to sulfadiazine in the presence of Daraprim. Sulfadiazine-resistant strains, on the other hand, responded much more dramatically to the potentiation effect of Daraprim (6 to 15 times).

DISCUSSION

The hypothesis upon which this investigation is based is depicted in Figure 1. Sulfadiazine competes with paramino-benzoic acid for enzyme systems involved in the formation of folinic acid because of the similarity in molecular structure of sulfadiazine and paramino-benzoic acid. The sulfadiazine/paramino-benzoic acid ratio which was depicted in Table 3 is the reverse of the Wood-Fildes ratio (1) and demonstrates that resistant strains require more sulfadiazine per unit of paramino-benzoic acid to initiate inhibition. This would be interpreted to mean that either more PABA is produced or PABA is more effectively utilized by the resistance strains of meningococci. Which of these explanations may be responsible for meningococcal (sulfadiazine) resistance cannot be determined from this data. An alternate interpretation of these findings is that the metabolic pathways in the formation of one carbon molecule by resistant strains are different than that of the sensitive strains. Some evidence for the latter was found in the Daraprim experiments where Daraprim was more effective as a sulfadiazine potentiator with resistant strains as shown in Table 4. Strains of Neisseria meningitidis, which were originally very resistant to sulfadiazine, became sensitive using concentrations of Daraprim from 1.5 μg/ml to 100 μg/ml. Also, as can be seen,

resistant strains demonstrated a greater potentiation by Daraprim than did the sensitive strains. It is possible that a second pathway exists, the "X-route", which is used more extensively by resistance strains. Figure 1 demonstrates a possible site of action whereby Daraprim blocks the "X-route", thus requiring the synthesis of one carbon moieties by way of the paramino-benzoic acid-folic acid pathway the same as the sensitive strains. With further study, it is possible that the combination of sulfadiazine and Daraprim could be used to control the nasopharyngeal carrier of sulfadiazine resistance meningococci.

TABLE 1. -- Fold Increase in Resistance of Meningococcal Strains in the Presence of Various Concentrations of Antagonist or End Products Dependent on Folinic Acid Synthesis

	Fold increase
PABA	10 to 26
Folic acid	none
L-Serine	none
Adenine sulfate	None to 5

TABLE 2. -- Sulfadiazine Sensitivity in Presence of Various Concentration of Paramino-benzoic Acid

Original sulfa sensitivity	PABA (1×10^{-6} M) 0.79	1.58	7.9	79.9
		Sensitive		
0.5 mg/100	1.0	3.0	5.0	>20
1.0 "	1.0	1.0	3.0	10
1.0 "	1.0	3.0	10.0	60
		Resistant		
3.0 "	10	40	>100	>100
3.0 "	3.0	3.0	10.0	>20
3.0 "	15.0	>20	>20	>20
3.0 "	10.0	40	80	>100
10.0 "	>20.0	>20	>20	>20
10.0 "	20	100	>100	>100
15.0 "	40	>100	>100	>100

TABLE 3. -- Ratio of Moles of Sulfadiazine/Mole of PABA at the Sensitivity Endpoint

Original sulfa-sensitivity	PABA (1×10^{-6} M) 0.79	1.58	7.9	79.9
		Sensitive		
0.5 mg/100	50	75	25	--
1.0 "	50	25	15	5
1.0 "	50	75	50	30
		Resistant		
3.0 "	151	75	50	--
3.0 "	758	-	-	--
3.0 "	805	101	404	--
3.0 "	505	1011	-	--
10.0 "	1151	2528	-	--
15.0 "	2023	-	-	--

TABLE 4. -- Differentiation of Sulfa-resistant from Sulfa-sensitive Meningococci by Response to Potentiation by Daraprim

Meningococcal strain	Fold increase in sensitivity to sulfadiazine
Sulfa-sensitive	3 to 6
Sulfa-resistant	6 to 15

POINTS IN METABOLIC PATHWAYS WHERE SULFADIAZINE AND DARAPRIM EXERT THEIR INHIBITORY EFFECT

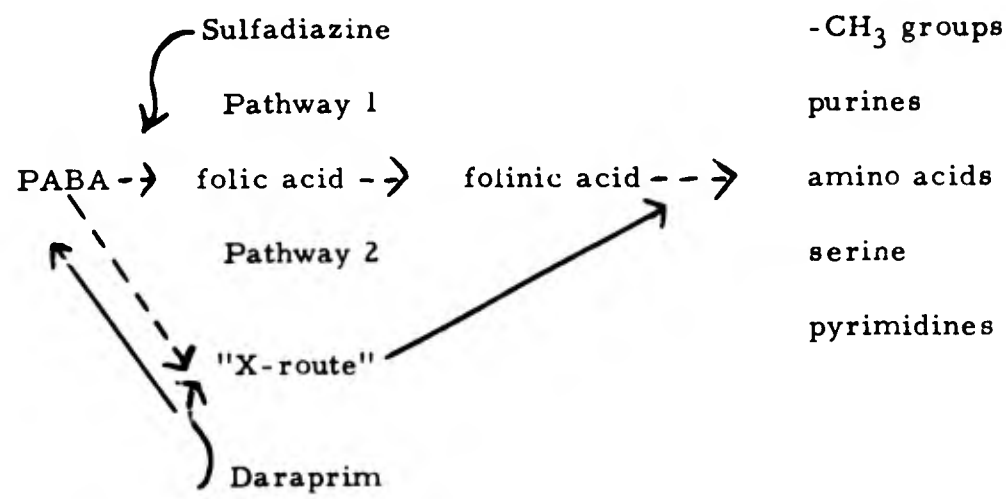

FIGURE 1

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RELATION OF SERUM FACTORS TO STRESS AND TO SUSCEPTIBILITY TO ACUTE RESPIRATORY DISEASES

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The purpose of this study was to further investigate what serum factors allow one individual to have a native resistance or natural immunity to microorganisms causing upper respiratory infection, while another individual was without this resistance (1, 2). Included in this project were measurements of serum haptoglobins, total proteins, and the lactate dehydrogenase isoenzymes patterns. These measurements were performed before and after a physical stressing condition, and again repeated after a 2-week interval. It was hoped that these parameters might be helpful in furthering our understanding as to the role of stress and susceptibility to infections.

MATERIALS AND METHODS

Approximately 80 recruits were bled within 72 hours after their arrival at Great Lakes. After they had been stressed (calisthenics) for 10 minutes, they were bled again. This same procedure was repeated in two weeks. The collected blood was centrifuged, and the serum frozen for later use.

Measurement of total protein: One drop of thawed sera was placed in the receptacle of American Optical Company's TS meter for the protein

determination. The total protein value was then read from the appropriate scale.

Haptoglobin concentration: The micro immuno agar diffusion test, as described by Ouchterlony, was carried out on a double-diffusion in a 1 mm thick agar gel.

Preparation of sera: The sera was diluted with distilled H_2O, to the following ration: 1:1 -- 1:512. All but the first two dilutions were used.

Preparation of plate: Clean microscope slides were covered with special agar (Noble) (1 gm to 100 ml d H_2O) and allowed to harden. A series of sample wells were punched into the hardened slabs and approximately 0.05 cc of the sample was dropped into the well. Anti-haptoglobin antisera was placed in the center well. The slab was then placed in an incubator at 37°C. During the incubation, the antigens and antibodies diffuse toward each other in the semisolid gel; and where they meet, arcs of visible precipitation are formed at the position where a certain concentration ratio of antigen to antibody exists. These plates were read at 24 and 48 hours. A standard curve (see Fig. 1) was derived by using known hyperhaptoglobin serum specimens as determined by the method of Jayle (3).

Lactate dehydrogenase isozymes: The agarose electrophoresis film set was used. This consisted of pre-made gels, sodium lactate and coenzyme NAD+ (nicotinamide adenine dinucleotide). The buffer was prepared according to the following specifications: 20.62 gm barbital (sodium barbital), 0.7 gm EDTA Na_2, volume brought to 2 liters with distilled H_2O, then 10 ml 1 N HCl to pH 8.8 were added. The sample wells were filled with 0.8 - 1.0 µl of sera using a microliter sample of dispension syringe (Turner No. 310-101), and disposable sample loading tips (Turner No. 301-011). The prepared agarose film was then placed face down in an electrophoresis cell with the sample wells nearest the cathode side. The conditions for high resolution were 120 v and a running time of 60 minutes. After the run, the film was treated with a mixture of NAD^+ - Na lactate and then placed in 37°C water bath for 10 min. The outer sample wells were then loaded with 1 µl of reduced nicotinamide adenine dinucleotide disodium salt, NADH (1 mg/ml buffer). The gel was next dried in an oven for 15 minutes at 85°C. By this technique, the serum lactate dehydrogenase (LDH) is separated into 5 fractions (isoenzymes) which appear as blue-white fluorescent lines underneath ultraviolet light (365 mµ). The amount of fluorescent reduced coenzyme NADH formed in the enzymatic reaction is proportional to the amount of lactate converted to pyruvate. The reaction is lactate + non-fluorescent $\xrightarrow{LDH}$ pyruvate and fluorescent. CH_3 CHOHCOO-HAD+ $\xrightarrow{LDH}$ CH_3 $COCOO^-$ + NADH + H^+. This permits calculation of LDH units in terms of µM NADH/10 min/100 ml serum. The fluorescence of

NADH is measured in the Turner Model 111 fluorometer equipped with a strip-scanning automated reader door. The filters used were primary = No. 48 + 2A, secondary = 7-60 with the sensitivity set at 30X. The results were automatically recorded on a Rustrak integrating recorder.

RESULTS

Table 1 shows the results of the total protein (TP) analysis. Using the initial total protein analysis as the basic standard for each man, three comparisons are made. Using these, the men are then placed into categories. Stress 1 refers to the comparison between the initial blood sample and the blood sample after stress on the same day. Stress 2 refers to the comparison between the initial blood sample after 2 weeks, and the blood sample after stress on that day. Initial refers to the comparison between the initial blood sample and the initial blood sample after 2 weeks. The (+) means that the TP has increased. The (N) means that the TP has decreased or remained the same.

Table 2 shows the responses of the haptoglobin during the trial. The stress 1, stress 2, and initial are the same as in Table 1. Increased haptoglobins occurred more frequently at 14 days in each of the illness categories. Significant increased levels of 175 mg/100 or greater also were more prevalent 2 weeks after the company was formed. The clinical significance of elevations in serum LDH isoenzymes is shown in Figure 2. (From Analytical Chemists, Inc.).

Table 3 shows a typical assay of the serum isoenzymes of LDH illustrating the enzymatic activity associated with each fraction. Also, noted is the presence of an additional fraction in those recruits, the recruit numbers which are underscored, with a respiratory illness. Figure 3 shows the serum coenzyme patterns of the well recruit, whereas, Figure 4 shows the patterns of those recruits who had respiratory illness.

Table 4 shows the recruit with reference to the sample, isoenzymes, and isoenzymes concentration present.

Sample 1 = initial blood
Sample 2 = initial blood after exercise
Sample 3 = initial blood 2 weeks later
Sample 4 = initial blood after exercise 2 weeks later
I = Isoenzyme number (from anode to cathode); percent total = the percentage of the entire isoenzyme concentration of that fraction.
Units LDH = μM NADH/10 min/100 ml sera.
Underscored recruit number = recruit reported to sick call.
Underscored units of LDH activity = value is above normal for that particular fraction

DISCUSSION

In viewing the total protein determination, no definite pattern can be found that will distinguish a susceptible from a non-susceptible on the basis of the initial stress. Increases of total protein were noted more frequently in illness groups following the second stress condition (14 days) than were noted in the group without illness. The same conclusion can be made in viewing the haptoglobin concentration data. In examining the two tables together and trying to correlate the two, one is not able to find a set pattern that will distinguish the susceptible from the non-susceptible. However, important factors that must be considered are: (1) the unusual low incidence of illness during the study; (2) the duration and type of stressing may not have been sufficient to stimulate a response, and (3) the time the specimens were obtained following the stressing.

Difficulties were encountered, also, in the interpretation of the haptoglobin determinations. Haptoglobin was studied as a possible stress parameter. There might be a problem with the definition of stress. In our study, muscular exercise (of 10 minutes duration) was considered to be stressful. It seems that exercise in this amount is a natural and not necessarily a stressful maneuver. Duration of activity is important. Swaiman and Awad (4) subjected human volunteers to strenuous controlled exercise over a 10-minute period and did not observe any significant increase in serum concentration of CPK, SGOT, SGPT, SLDH, MDH, or aldolase. They concluded the exercise was not sufficient in duration or severity to produce some changes reported in the literature (5). Perhaps this is the same case with the serum haptoglobin.

There are a number of problems with obtaining significant findings with haptoglobin as the variable. There is much variation in haptoglobin from one individual to the next, while some individuals completely lack detectable haptoglobin (6). Also, it has been shown that haptoglobin may occur in four different forms in the population, and specific variations may occur in 10 --- 55% of a given population (6). Allison, et al., studied a Nigerian population and found no detectable haptoglobin in 32%. Possibly though, this absence was due to a chronic or recent acute hemolysis (6). However, this absence questions the importance of haptoglobin in iron conversion or haptoglobin loss, and, therefore, in stress.

Comparing patterns of rise and fall changes in the LDH isoenzymes

and comparing the sum totals of the LDH units gives no significant pattern for predicting natural immunity. In those cases where there was an increase of the isoenzyme percent above normal, no general conclusion could be drawn due to its random appearance in the sample population. It is noteworthy to remark that of those individuals that did report to sick call, all showed an extra peak in the isoenzyme pattern prior to the number 1 peak (number from anode to cathode). We are not, at this time, prepared to state just what it was that caused this, but further investigation may bring this to light as a stress parameter.

Another observation pertaining to incidence of disease with stress concerns the general adaptation syndrome. This syndrome shows that if an individual is stressed over prolonged periods, at first his susceptibility to a second stress will increase for a brief period, then he will go through a fairly long period in which he is actually less susceptible, then a period comes in which the susceptibility increases greatly (6). Perhaps this explains the phenomenon seen in recruits who have a great number of upper respiratory illness at 2 - 3 weeks. The primary stress in this case would be mostly emotional due to change in environment, etc., and the second stress would be the virus causing the disorder. A physical (muscular) stress would probably have to be eliminated since the recruits would become better conditioned as training proceeded, and exercise then could not be called a stress factor.

Our method of determining illness needs further revision. It is a well known fact that not every recruit who developes an URI will report to sick call. Since they did not, we, at this time, have no method of including them in the study. Perhaps these results will be more meaningful after serology to detect infection is done on their blood samples.

SUMMARY

Total protein and serum haptoglobin concentration taken together and separately do not seem to be a factor with stress in natural immunity to disease. Lactate dehydrogenase isoenzyme patterns vary within an added peak in those individuals with reported sickness.

TABLE 1 -- Alterations in Total Serum Protein Levels Correlated with Illness following Physical Stress

Number Subjects		Percent of each Stress Categories*							
		+++	++N	+NN	NNN	NN+	N++	N+N	+N+
No illness	61	13	46	5	0	3	0	26	5
With illness	16	6	25	6	6	0	0	43	12
Total	77	14	42	5	1	3	0	30	7

*First + = increase in total protein following the first stress

Second + = increase in total protein following the second stress

Third + = increase in total protein between the initial specimen and the blood sample after 2 weeks

N = Decrease or no change

TABLE 2 -- Increase in Serum Haptoglobins Before and After Stressing Correlated with Various Illness Categories

Illness Category	Number in Group	Time and Conditions when Specimens were Obtained: Day 1 Before	Day 1 After (Stress)	Day 14 Before	Day 14 After (Stress)	Total
URI *	15		20.0	53.0	6.5	80.0
NURI **	15		6.5	46.0	0.0	53.0
Other	40		17.5	52.0	28.0	70.0
Total	70		13.0	61.0	18.0	82.0

Significant Increased Levels*** of Haptoglobin Before and After Stressing in Various Illness Categories

URI *	15	6.5	6.5	20.0	13.0
NURI **	15	6.5	0.0	33.0	40.0
Other	40	12.5	10.0	35.0	45.0
Total	70	10.0	7.0	31.5	37.0

* Upper Respiratory Illness

** NURI - Non-Upper Respiratory Illness

*** Significant increased levels 175 mg% or greater

TABLE 3 -- Relative Value, Per Cent Observed in the LDH Isoenzyme

Recruit Number	Sample Number	Patterns 0	1	2	3	4	5
717	1		22.0	29.8	20.7	16.8	10.3
	2		23.0	29.2	20.0	15.3	12.3
	3		25.0	35.7	19.6	8.9	10.7
	4		28.5	28.5	14.2	7.1	21.4
730	1		26.3	31.5	21.0	14.4	6.5
	2		20.6	26.9	26.9	15.8	9.5
	3		24.6	31.5	24.6	12.3	6.8
	4		23.2	32.5	20.9	9.3	13.9
731	1		24.7	32.9	23.5	11.7	7.0
	2		25.7	33.3	24.2	12.1	4.5
	3		21.1	35.5	24.4	11.1	7.7
	4		0	75	15	9	10
763	1		19.2	36.1	25.3	10.8	8.4
	2		23.5	29.4	29.4	11.7	5.8
	3		18.2	45.4	18.2	9.0	9.0
	4		15.5	25.2	31.0	19.4	8.7
778	1	10.0	18.7	32.1	20.1	12.8	5.5
	2	12.2	20.0	31.0	20.0	12.2	4.4
	3	12.5	22.9	30.2	17.7	9.3	7.2
	4	7.6	15.3	38.4	18.4	12.3	7.6
787	1	10.0	21.0	31.0	21.0	10.0	6.7
	2		16.3	34.4	26.2	14.7	8.1
	3		18	31.9	27.7	13.8	8.3
	4		18.5	30.8	24.6	14.8	11.1
801	1		15.5	32.1	25.6	16.5	10.0
	2		17.3	29.3	25.3	13.3	14.6
	3		23.8	32.3	24.7	13.3	5.7
	4		18.5	31.9	23.7	15.4	10.3
802	1	7.1	19.6	35.7	26.7	7.1	3.5
	2		22.2	37.0	24.0	11.1	5.5
	3		29.4	31.3	25.4	7.8	5.8
	4	4.9	20.9	33.3	24.6	11.1	4.9
804	1		22	31	23	14	10
	2		22.8	31.4	25.7	11.4	8.5
	3	7.3	22.0	35.2	27.9	5.8	1.4
	4		21.3	39.3	22.9	8.1	8.1

Underscored figures are abnormal

TABLE 4 -- Absolute Values for Serum Isoenzymes found in Naval Recruits

Recruit Number	Sample Number	Units LDH in Isoenzymes - μM NADH/10 min/100 ml. Sera 0	1	2	3	4	5	Total
717	1		66.4	89.9	62.4	50.7	31.1	300.5
	2		51.1	64.9	44.5	34.0	27.3	231.8
	3		35.0	50.0	27.4	12.5	14.9	139.8
	4		41.0	41.0	20.4	10.2	30.8	142.7
730	1		78.4	93.9	62.6	42.9	19.4	297.1
	2		50.9	66.4	66.4	39.0	23.5	246.2
	3		70.4	90.1	70.4	35.1	19.4	295.4
	4		38.9	54.6	35.1	15.6	23.3	169.5
731	1		82.3	109.6	78.3	39.0	23.3	332.5
	2		66.3	85.9	62.4	31.2	11.6	257.4
	3		52.8	88.8	61.0	27.8	19.2	247.6
	4		0	58.7	11.7	0	7.8	
763	1		56.	105.4	73.8	31.5	24.5	291.2
	2		42.3	52.9	52.9	21.0	10.4	179.5
	3		40.3	100.6	40.3	19.9	19.9	220.5
	4		40.0	65.0	71.6	44.8	20.0	241.4
778	1	39.4	73.7	126.5	79.2	50.4	21.7	390.5
	2	39.7	65.0	100	65.	39.7	14.3	323.7
	3	42.3	77.5	102.2	59.9	31.5	24.3	337.7
	4	19.9	40	100.8	48.2	32.2	19.9	261.0
787	1	42.	88.2	130.2	88.2	42	28.1	157.7
	2		41.1	86.1	66.0	37.0	20.4	250.6
	3		60.9	107.9	66.0	46.7	28.1	309.6
	4		66	109.9	87.8	52.8	39.6	350.1
801	1		64.2	132.9	105.9	68.3	41.4	412.7
	2		52.2	88.4	76.3	40.1	44	301.0
	3		88.3	119.8	91.6	49.3	21.1	370.1
	4		46.1	79.4	59	38.3	25.6	248.4
802	1	16	44.2	80.5	60.2	16	7.9	208.8
	2		48.3	80.4	52.2	24.1	11.9	216.9
	3		60.4	64.3	52.2	16.0	11.9	204.8
	4	16	68.2	108.6	80.2	36.2	16.0	309.2
804	1		77.6	109.3	81.1	49.4	35.3	352.2
	2		56.2	77.5	63.4	28.1	20.9	246.2
	3	17.5	52.7	84.4	66.9	13.9	3.4	221.3
	4		45.8	84.5	49.2	17.4	17.4	214.4

Underscored figures are abnormal

HAPTOGLOBIN CONCENTRATIONS AS DETERMINED BY AN AGAR DIFFUSION MICRO IMMUNO METHOD

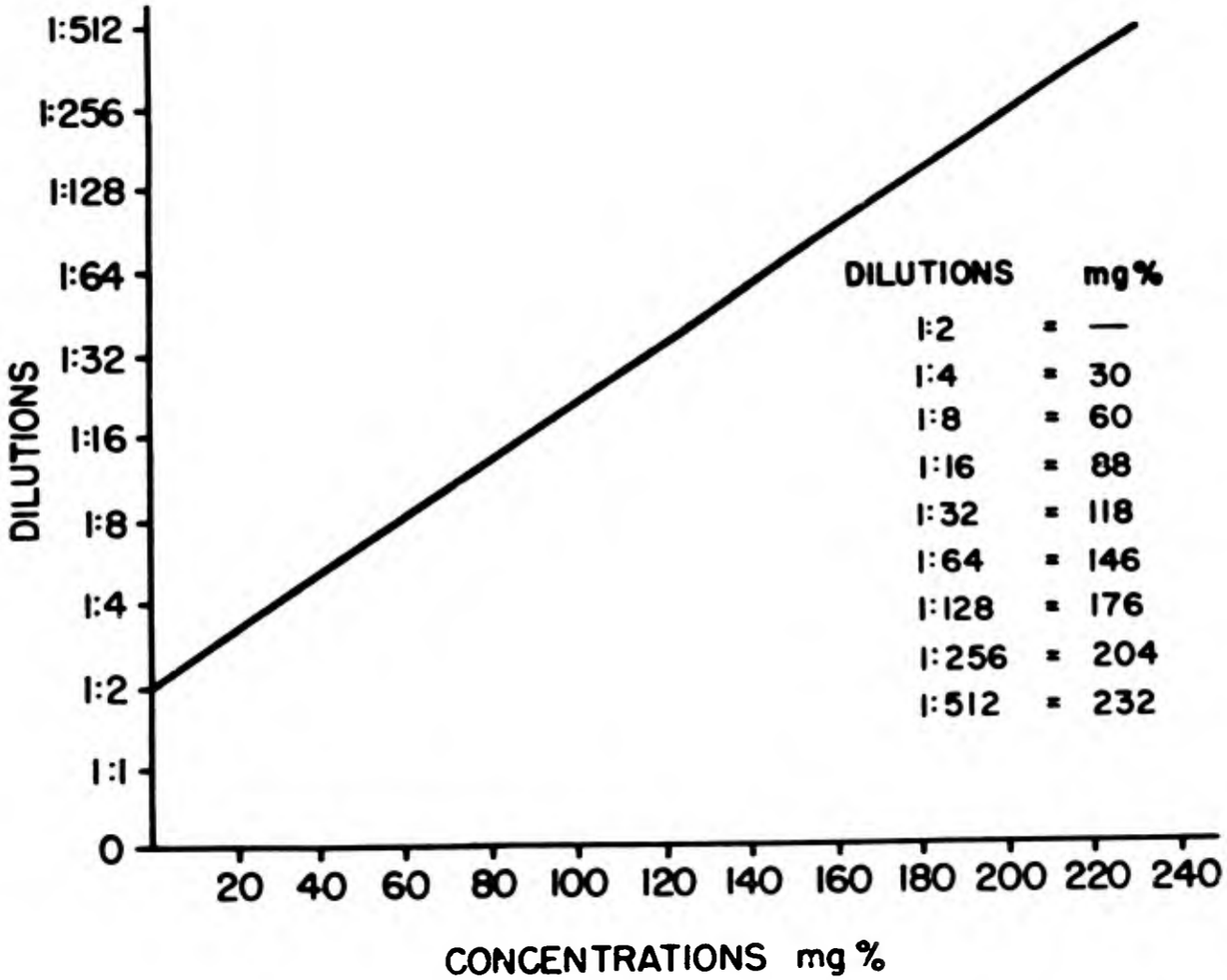

FIGURE 1

CLINICAL SIGNIFICANCE

SERUM LACTATE DEHYDROGENASE ISOENZYMES

LDH* ISOENZYME	NORMAL RANGE (% of total)	INTERPRETATION **	
		INCREASED	DECREASED
1 2	17-27% 28-38%	Myocardial infarction Hemolysis Leukemia Pernicious anemia Adenocarcinoma of the colon Testicular carcinoma Renal tubular necrosis ± Pulmonary infarction	
3	19-27%	± Pulmonary infarction With LDH 1 and LDH 2 in renal tubular necrosis	
4	5-16%	With LDH 5 in hepatic necrosis, renal tubular and cortical necrosis, and certain malignant tumors	
5	5-16%	With LDH 3 and LDH 4 in infectious mononucleosis Hepatic congestion Hepatitis Renal cortical necrosis Prostatic adenocarcinoma Skeletal muscle necrosis Dermatomyositis Testicular carcinoma Multiple myeloma Renal cell carcinoma	Muscular dystrophy Certain cases of neurogenic muscular atrophy

* Numbering from anode to cathode

** The frequency of abnormal LDH isoenzyme patterns in the listed conditions has not been established, nor is the list intended to be complete. Reference: Elevitch, F.R. in Progress in Clinical Pathology, Stefanini, M., Ed., Grune and Stratton, New York, 1966.

FIGURE 2


SERUM LACTATE DEHYDROGENASE
ISOENZYME PATTERN
HEALTHY RECRUIT
BEFORE
AFTER STRESS
UM NADH / 10 MIN / 100 ML SERUM
40
35
30
25
20
15
10
5
0
STD 5 4 3 2 1 0
STD 5 4 3 2 1 0

FIGURE 3

SERUM LACTATE DEHYDROGENASE
ISOENZYME PATTERN
RECRUIT WITH AN UPPER RESPIRATORY
INFECTION

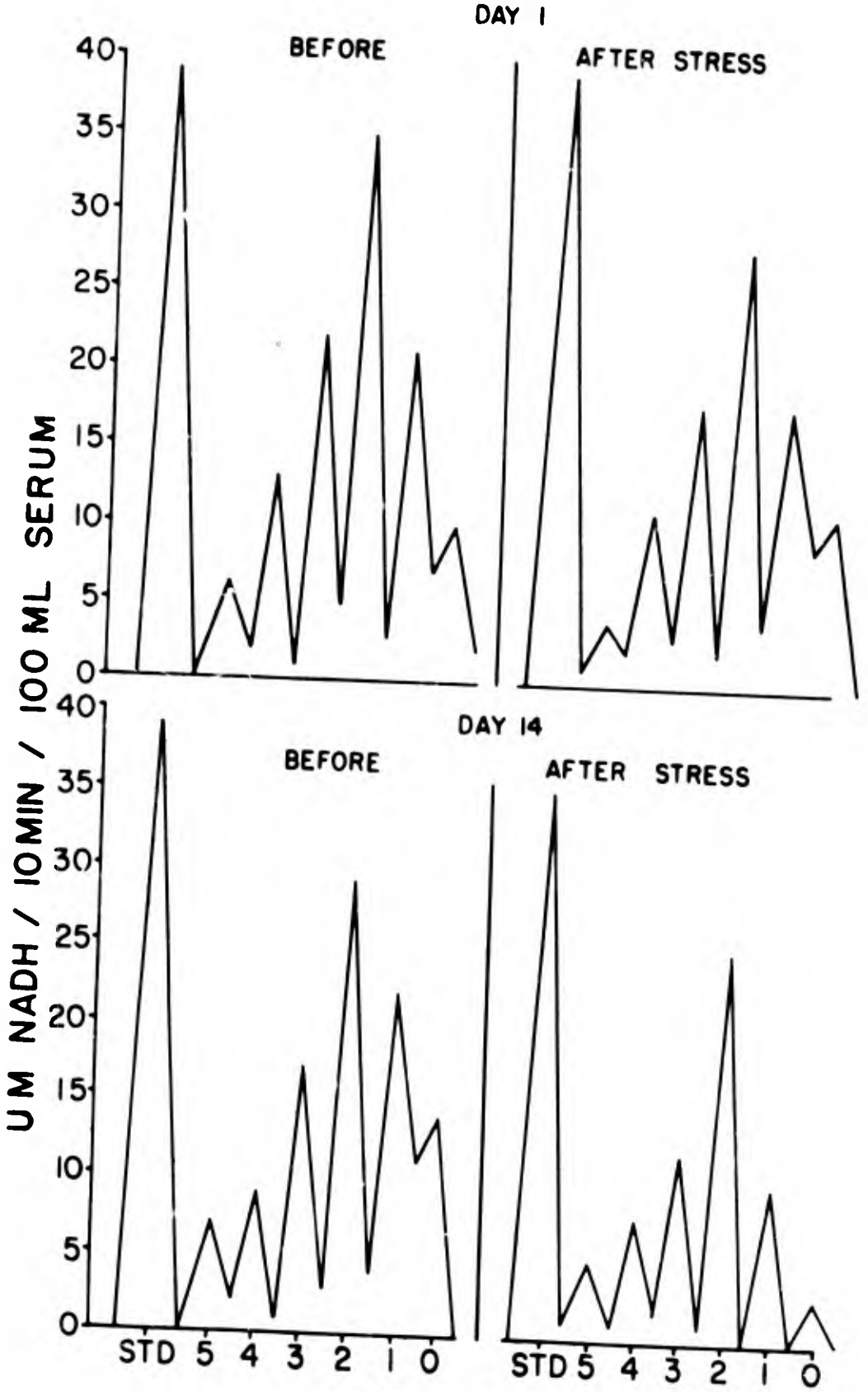

FIGURE 4

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THE EFFECTS OF ROUTINE IMMUNIZATION ON THE INTERFERON LEVELS IN THE BLOOD FRACTIONS OF NAVY RECRUITS

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In recent years, it has been discovered that the establishment of resistance to viral infection is often accompanied by the synthesis of a substance in the cell known as interferon. It has further been shown that this substance does not directly inactivate the virus; but, rather, it inhibits the intracellular replication of the virus. Moreover, interferon in vitro has been shown to exhibit relative specificity in that it is more effective in protecting those cells in which it is produced than in heterospecific cells (1).

Some of the physiochemical properties of interferon included in its characterization are: (a) a small, nondialyzable protein molecule inactivated by proteolytic enzymes, including trypsin; (b) relatively stable to heat; (c) stable at high and low pH, and (d) having no proteolytic or nuclease activities (1).

Further investigation into its mode of action has led to a number of different hypotheses. However, they are all based on the well documented experiments which prove that interferon acts by blocking formation of new viral RNA.

It has also been suggested that interferon induces the formation of a new protein which inhibits virus production; and the formation of this protein is of cellular origin and mediated by a DNA-dependent RNA (2). Evidence for the necessity of a DNA-dependent RNA system in the action of interferon is derived from a variety of experiments which prove conclusively that the inhibitory action of interferon to a virus challenge is itself inhibited by treatment with actinomycin-D; and the primary action of actinomycin is to prevent the synthesis of RNA on a DNA template (3).

Other experiments have shown that not only is viral RNA synthesis inhibited by interferon, but also host cell RNA. This led to the hypothesis that interferon may be responsible for the production of some type of unstable enzyme system which degrades newly synthesized RNA (4).

Further evidence for the necessity of protein synthesis in interferon activity is derived from experiments showing the effects of p-fluorophenylalanine (FPA) (5). FPA is incorporated into proteins during their synthesis and leads to a reduction in biological activity of these proteins. Using the percentage inhibition of plaque formation as an index of interferon action, it has been shown that treatment with FPA will inhibit the antiviral activity of interferon.

In any case, the precise mechanism of interferon action remains to be proven. What is known is that interferon is a protein, and its mode of action involves the inhibition of viral RNA replication.

The importance of interferon studies with regard to the military arises from the fact that viral infections among military recruits have proven to be an expensive and time-consuming problem during the training period. At Great Lakes Naval Training Center, it has been observed that the greatest incidence of viral infections, especially acute respiratory diseases, occurs during the third and fourth weeks of training. This has been the period during which recruits receive most of their routine immunizations. Observations by the Naval Medical Research Unit No. 4, in a study of approximately 65,000 recruits over an 18-month period, have shown that delayed immunization has a beneficial effect in reducing the incidence of acute respiratory disease in all phases of training in every month of the year (6). It had also been shown in other laboratories that there is a hyporeactivity of interferon after endotoxin immunizations of animals (7).

It is the purpose of this study to determine the interferon levels in the blood fractions of Navy recruits before and after routine immunizations received during their training period in order to evaluate whether they are altered significantly by such procedures.

MATERIALS AND METHODS

Navy recruits at NTC, Great Lakes, Illinois, receive six series of prophylactic procedures over an 8-week period (Table 1).

The blood specimens of 15 men from a company of recruits receiving these immunizations were taken prior to each immunization and 48 hours after each immunization. The blood specimens were processed, and interferon assays were done on the serum, plasma, and blood cells of 10 men after each treatment.

In addition, 15 men were bled before and 4 hours after receiving the

typhoid-paratyphoid vaccine and its booster (3 weeks later). Another 15 men were bled on the third, fifth, and sixth days after receiving yellow fever and cholera vaccines.

The method of interferon assay (Table 2) is a modification of that described by Wheelock (8). The specimens to be tested for interferon were diluted in phosphate-buffered saline, and 0.025 ml of each dilution was added to 1-day-old cultures of human fetal lung cells grown in microplates (9). After a 20-24-hour incubation at 37°C, the cultures were washed with 0.1 ml of phosphate-buffered saline. Another 0.025 ml of diluent was added to each plate, and then 0.025 ml of cold growth medium containing sufficient tissue culture infective doses of Sindbis virus to produce 75% degeneration within 24 hours. Cultures were considered to be protected when there was 50% less cell degeneration in test than in the control.

RESULTS

1. Incidence of Interferon Detected in the Various Blood Fractions Assayed.

A total of 178 blood specimens were processed, and interferon assays were done on the serum, plasma, and blood cells from each specimen. Interferon was detected in 10.1% (18/178) of the serum preparations, 10% (16/160) of the plasma preparations, and 10% (16/160) of the blood cell preparations (Table 3).

2. Effect of Treatment on Interferon Levels.

No interferon was detected in the specimens taken before and after the diphtheria, tetanus and influenza vaccines; the penicillin prophylactic treatment; or tuberculin skin test. After the typhoid-paratyphoid and typhus vaccines, 30% of the specimens showed a decrease after 4 hours, while 70% remained unchanged; and 20% showed a decrease after 48 hours, while 80% remained unchanged. There were no increases in interferon titers.

After the typhoid-paratyphoid and typhus boosters were given, in combination with the live polio vaccine and cholera booster, 10% showed an increase after 4 hours, 30% showed a decrease and 60% remained unchanged. After 48 hours, there were no increases, while 20% decreased and 80% remained unchanged.

The results of all typhoid-paratyphoid and typhus vaccinations showed

that 10 of 40 cases (25%) had a decrease in interferon levels after inoculation; and only 1 of 40 cases (2.5%) had an increase. This one case received the typhoid-paratyphoid and typhus in combination with the polio and cholera immunizations. Thus, it cannot be said which of these latter treatments caused the increase.

After the yellow fever vaccine, there were no increases after 48 hours, while 10% decreased and 90% remained unchanged. In another group of specimens, which were taken on the third, fifth, and sixth days after inoculation, 33% increased, 33% decreased, and 33% remained unchanged on the fifth day with respect to the third day readings. On the sixth day, there were no increases, while 50% decreased and 50% remained unchanged with respect to the fifth day (Table 4).

DISCUSSION

As can be seen from the data on interferon determinations in the various blood fractions, the total summation does not seem to present any difference in the incidence of detection in the serum, plasma, or blood cells. Upon closer analysis, however, it can be seen that the interferon levels in the serum taken from those receiving the typhoid-paratyphoid and typhus vaccines decreases from 8 cases for the first inoculation to 2 cases for the booster; while, for the same group, the incidence in the blood cells rises from zero cases for the first inoculation to 13 cases for the booster. The differences in the detection of serum interferon between the first and second inoculation barely approaches statistical significance with a P value of less than 0.05; whereas, the difference in the detection of interferon in the blood cells definitely is statistically significant with a P value of less than 0.01. Also, after the yellow fever vaccine the incidence of detection is much higher in the serum than in either the plasma or the blood cells. Whether these results are due to the technique or to some phenomenon resulting from the immunizations remains to be studied.

With regard to the effects of treatment on the interferon levels, it is noteworthy that there are many more cases which show a decrease in status, rather than an increase, primarily after the typhoid-paratyphoid and typhus immunizations. Whether or not this can be related to the findings of Pierce and Edwards (6), as to the beneficial effect of delayed immunizations, remains to be proven; but, the data is not inconsistent

with the hypothesis of decreased host resistance due to immunization.

It is obvious that the number of cases from which positive results were obtained is not large enough for any definite conclusions. One of the main problems to be solved in the technique is the determination of the proper time interval post-inoculation at which the interferon titer will be highest in the blood stream. There have been varying reports in the literature as to the time after infection that the interferon titer reaches its peak; and it is very likely that it will be different for each antigen. Another factor to be considered in the determination of this interval is the physiological nature of the population being used. Navy recruits lead a more active life than a person from the general population, and this increased metabolism may affect the clearing of interferon from his blood stream.

It is hoped that such studies of more definite, quantitative nature will be continued to determine the effect of immunization on host resistance as measured by the presence or reduction of interferon.

SUMMARY

Interferon assays were done on the serum, plasma, and blood cells of 178 specimens taken from 86 Naval recruits receiving routine immunizations over an 8-week period, in order to determine whether such procedures alter the interferon levels.

It was found that: 1) The incidence of interferon detection in the serum, plasma, and blood cells did not differ in the total results; but, there was a decrease in the number of cases showing interferon levels in the serum and an increase in the number of cases showing interferon in the blood cells after the endotoxin vaccination booster, and 2) There were more cases showing a decrease, rather than an increase, in interferon levels after the typhoid-paratyphoid and typhus immunizations.

It is suggested that more work be done on the determination of the proper time interval after vaccination at which the interferon titer will be at its peak; and it is felt that the indication of this study justifies its continuation on a larger scale.

TABLE 1 -- Routine Immunizations, Prophylaxis, and Skin Tests Given to Naval Recruits

Training Week	Treatment
1	Sulfadiazine; influenza vaccine; diphtheria and tetanus vaccines.
2	Benzathine penicillin G; tuberculin skin test (PPD, intermediate strength).
3	Typhoid-paratyphoid vaccine; typhus vaccine.
5	Yellow fever vaccine; cholera vaccine.
6	Diphtheria and tetanus booster; smallpox vaccination.
8	Polio polyvalent vaccine; typhoid-paratyphoid booster; typhus booster; cholera booster.

TABLE 2 -- Determination of Interferon

Specimen	Tissue culture and Pretreatment		Test		Presence of Interferon
a) Sera b) Plasma c) Blood cells (white & red)	Human embryonic lung cells with blood fraction	Incubate 20-24 hrs at 37°C	Add Sindbis virus (5000 $TCID_{50}$) (or sufficient virus to produce 75% CPE in 24 hrs)	Incubate 20-24 hrs at 37°C	50% less CPE in test than in virus control

TABLE 3 -- Results of Interferon Determination on Recruit Blood Fractions

Treatment	Serum (1:10)	Plasma (1:10)	Blood Cells (1:50)
Diphtheria and tetanus vaccine; influenza vaccine	0 (0/20)	0 (0/20)	0 (0/20)
Benzathine penicillin G; tuberculin skin test	0 (0/20)	0 (0/20)	0 (0/20)
Typhoid-paratyphoid vaccine; typhus vaccine	*20% (8/40)	15% (6/40)	**0 (0/40)
Yellow fever vaccine; cholera vaccine	21% (8/38)	0 (0/20)	10% (2/20)
Diphtheria and tetanus booster; smallpox vaccination	0 (0/20)	0 (0/20)	5% (1/20)
Polio polyvalent vaccine; typhoid-paratyphoid booster; typhus booster; cholera booster	*5% (2/40)	25% (10/40)	**32% (13/40)
Totals	10.1% (18/178)	10% (16/160)	10% (16/160)

*P = < 0.05

*P = < 0.01

TABLE 4 -- Effect of Treatment on Interferon Levels

Treatment	Interval	Percent with change in interferon status: Increase	Decrease	No change
Diphtheria and tetanus vaccine; influenza vaccine	24 hrs	0	0	100 (no titer)
Benzathine penicillin G; tuberculin skin test	48 hrs	0	0	100 (no titer)
Typhoid-paratyphoid vaccine; typhus vaccine	4 hrs	0	30 (3/10)	70 (7/10)
	48 hrs	0	20 (2/10)	80 (8/10)
Yellow fever vaccine; cholera vaccine	48 hrs	0	10 (1/10)	90 (9/10)
	3 days	-	-	-
	5 days	33 (2/6)	33 (2/6)	33 (2/6)
	6 days	0 (0/6)	50 (3/6)	50 (3/6)
Diphtheria and tetanus booster, smallpox vaccination	48 hrs	0	0	100 (no titer)
Polio polyvalent vaccine; typhoid-paratyphoid booster; typhus booster; cholera booster	4 hrs	10 (1/10)	30 (3/10)	60 (6/10)
	48 hrs	0	20 (2/10)	80 (8/10)

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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 1966. The titles of the projects are as follows:

1. The Effects of Grouping on Antibody Production and Death Rates among Mice.
2. The Effect of Penicillin on the Oral Mycoplasma Flora of Naval Recruits.
3. Studies on the Mechanisms of Sulfadiazine Resistance among Meningococci.
4. Relation of Serum Factors to Stress and to Susceptibility to Acute Respiratory Diseases.
5. The Effects of Routine Immunization on the Interferon Levels in the Blood Fractions of Navy Recruits.

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