U.S. NAVAL SUBMARINE MEDICAL CENTER
NAVAL SUBMARINE BASE NEW LONDON
GROTON, CONNECTICUT 06340

MEMORANDUM REPORT NO. 68-5

FLUORESCENT ANTIBODY TECHNIQUE IN THE RAPID IDENTIFICATION OF VIRAL AGENTS IN SUBMARINES

MF022.03.03-9025.28

22 March 1968

Investigator:

LT John A. Lee, MC, USN

Transmitted by:

LCDR T. N. Markham, MC, USN
Head, Military Operations Branch

Reviewed and Approved by:

Charles F. Gell, MD, DSc(Med)
Scientific Director

Approved and Released by:

Gerald J. Duffner
Captain, MC, U.S. Navy
Commanding Officer

SUMMARY PAGE

THE PROBLEM

To investigate the feasibility of utilizing fluorescent antibody technique as a means of rapid identification of viral agents in upper-respiratory outbreaks aboard a Fleet Ballistic Missile Submarine on patrol.

FINDINGS

This preliminary investigation established an indirect fluorescent antibody technique for use in the laboratory; its validity was demonstrated by in vitro study. Pending future successful demonstration of in vivo studies, the technique can be adapted as a screening test for specific infections on board an FBM.

APPLICATIONS

When fully developed, this technique could be a most useful tool in determining whether a small number of viruses are the causative agents of recurrent upper-respitatory infections within an FBM crew. And further use of the technique in studying specific viral infections may form the foundation for eventual application of this technique for rapid diagnosis of URI (upper-respiratory infection).

ADMINISTRATIVE INFORMATION

This investigation was conducted aboard the U.S.S. KAMAHAMEHA, when the author was serving as Medical Officer of the Gold Crew. It was submitted by him in partial fulfillment of requirements for qualification as a Qualified Submarine Medical Officer. It was selected for publication as a Submarine Medical Research Laboratory Report, designated as Report No. 518, MF022.03.03-9025.29, in order to make the material available in the literature of submarine medicine and for use in the School of Submarine Medicine at the Submarine Medical Center.

PUBLISHED BY THE NAVAL SUBMARINE MEDICAL CENTER

FLUORESCENT ANTIBODY TECHNIQUE IN THE RAPID IDENTIFICATION OF VIRAL AGENTS IN SUBMARINES

INTRODUCTION

Fleet Ballistic Missile (FBM) submarines, while on patrol, provide a unique situation in which the atmosphere is self-contained and the men are completely isolated from the outside environment. The submarine becomes a form of laboratory, in which many of the usual variables are either controlled or eliminated. Temperature and humidity are held relatively constant. The possibility of further introduction of pathogens while the submarine is submerged, is practically non-existent. The activity of the men, while on patrol is less varied, their contacts limited to their shipmates. This greatly facilitates the study of the environmental factors. For these reasons, an FBM submarine on patrol is ideally suited for the investigation of various aspects of the epidemiology of disease.

On reviewing the health and habitability data of the FBM patrols, one of the most significant features is the relatively high incidence of upper-respiratory infections (URI) and the occasional occurrence of the so called "mid-patrol syndrome". "Mid-patrol syndrome" is an out break of URI after several weeks at sea. These outbreaks are most intriguing in that the atmosphere of the ship has been completely isolated from the surrounding environment since the hatch was closed. These pathogens, believed to be viral, must have either a prolonged latent period of infection or, as has been suggested by Andrewes (1), be due to activation of latent infection. A number of viruses have been shown to persist at a low level in tissue cultures, viral synthesis and interferon-production being balanced so that the virus is neither visibly destructive nor wholly eliminated. It is proposed that in a community, numerous respiratory viruses are constantly being passed back and forth. They may be present in small foci of the mucous membrane, multiplying and being shed at a minimum level. The viruses are present in insufficient amounts to engender an effective general immunity or to permit ready detection by viral isolation technique. These local foci may develop into full scale infection when stress factors are introduced. It would seem entirely possible that the repeated occurrence of URI within one crew, while on patrol, is caused by a small number of viruses that are normally present among the crew. Isolation and identification of these agents would be most helpful in developing some type of serological test for rapid recognition in the event of an outbreak of URI in the previously non-infected individuals of the same crew.

Fluorescent antibody (FA) technique, since its introduction by Coons et al (2), has been used increasingly with success as an immunohistochemical tool. This technique is based on the same principles as are other immunological reactions involving antigens and antibodies. Antibodies are labeled with the fluorescein dye (fluorescein isothiocyanate). When the labeled antibodies come into contact with homologous antigens in tissue cells or smears, they are deposited onto the antigen sites. Excess, labeled material is then washed away. Examination of the slide under a fluorescent microscope would reveal the antigen sites emitting light of a different wavelength by the excited fluorescent dye.

Fluorescent antibody (FA) technique has been applied in the diagnosis of infections due to streptococci, Hemophilus influenzae, Bordetella pertussis, enteropathogenic Escherichia coli and gonococci (14). In the field of viral and rickettsial infections, it has been applied in the diagnosis of rabies with good results (10).

The advantages of this technique lie in its sensitivity and specificity. Its great sensitivity allows detection of antigen-antibody reaction at the cellular level. Since it is essentially a serologic stain, this technique has the same specificity as does serotyping. Its greatest advantage, however, lies in the relatively short time required to obtain results. We are dealing in terms of hours rather than the days and weeks that are required in tissue culture and serotyping. The author, therefore, investigated the possibility and feasibility of utilizing the FA technique as a means of rapid identification of viral agents in URI outbreaks on board an FBM on patrol.

METHOD AND EQUIPMENT

The isolation and identification of the etiologic viral agents in the URI in an FBM crew was carried out on board the FBM to which the author was attached (USS KAMAHAMEHA) (G). This study was conducted in conjunction with an ongoing epidemiological survey supported by the Naval Biological Laboratory, (Oakland, California). Since the space available on board a submarine for laboratory equipment and procedure is severely limited, miniaturized equipment and system for viral isolation developed by the staff of Naval Biological Laboratory and Naval Medical Research Unit #1 were employed. This included: A small freezer weighting about 60 lbs. and capable of maintaining -60°C; a portable roller drum incubator of 26 inches in diameter and capable of incubating either three 8-oz bottles, or about 200 screw-cap vials in a circular, honeycomb structure; and a system of tissue culture using half-dram screw-cap vials. The

equipment and supplies were all furnished by Naval Biological Laboratory (E and E Div.), Naval Supply Center, Oakland, California.

At the beginning of patrol, crew's assigned bunk numbers were located and indicated in the ship's diagram. Background questionnaires were filled out by each crew member. When a person developed acute respiratory infection, his signs and symptoms were recorded and throat swabs and serum samples collected. One throat swab was put in L-15 medium in a 1-dram vial and stored at -60°C. One throat swab washing was used to inoculate two cell lines of tissue cultures. The inoculated cultures were frozen and stored at -60°C when cytopathogenic effects (CPE) or cell deterioration occurred. Convalescent sera were collected at the end of three weeks and six weeks.

EMK and HEK cells were used for tissue cultures. After the cells were thawed, washed and resuspended, 0.5 ml of cell suspension was held at room temperature and was to be inoculated with 0.5 ml of throat swab washing within an hour. Control vials were prepared by adding 0.5 ml of L-15 with 2.5% fetal calf serum to the cell suspension. Control and inoculated tissue cultures were prepared in both cell lines. Vials were then incubated at 34-1°C in the roller drum incubator. The vials were observed at the end of 24 hours for toxicity. If deterioration was noted, the vials were frozen at -60°C and stored. After 3 to 5 days CPE may occur in the inoculated vials, while control vial cells remain normal. When CPE appeared, the vial was frozen and stored at -60°C. Some control cells persisted up to two weeks. When cell deterioration finally occurred, all vials were frozen and stored at -60°C.

At the end of patrol, all samples collected were flown to Naval Biological Laboratory, carefully packed in dry ice. Further processing of the samples was carried out at NBL by serial passage of the inoculated cells and serotyping of the isolate, wherever obtained.

Viruses isolated from the first patrol of the FBM crew are as follows:

Virus Isolated	Type	No. of Isolation	Serum Neutralization Paired Sera	Diag. Rise (4X)
Echo	28	3	23	5
Coxackie	A21	1	21	1

Results from the second patrol were still being processed at the time this report was prepared.

The study of the application of FA technique was carried out at Naval Biological Laboratory by the author during the off-crew period. An attempt was made to establish a laboratory model, both in vitro and in vivo, that was adaptable for use on board an FBM. An indirect fluorescent antibody technique (IFAT) was chosen. In the indirect technique, the fluorescein dye is conjugated to a species specific antigammaglobulin. When an unlabeled specific antiserum is layered over a tissue smear containing the antigen, an antibody-antigen complex is formed in situ. When the excess molecules are washed away, the fixed gammaglobulin is then detected by fluorescent antigammaglobulin serum.

IFAT is generally more sensitive than the direct method due to the favorable antibody to antigen ratio in immunological reactions. It also eliminates the necessity of labeling each individual antiserum for each antigen. When no suitable antigen is available for the preparation of antisera, the convalescent serum may be used.

In the in vitro study, rabbit kidney (RK) tissue culture of para-influenza virus was used as the antigen. The human convalescent sera titrated by compliment fixation (CF) were used as the antisera. The sera were pooled and filtered through a 0.22u millipore filter. Serum with CF titer of less than 1:8 was considered to be negative. Both positive and negative sera were used against RK tissue culture of para-influenza virus and normal cell control. Antihumangammaglobulin, conjugated with fluorescein isothiocyanate (FITC), was then used on these samples to test for fluorescence. In this study, an attempt was made not only to test the validity of the system, but also the specificity and the optimum dilution of antisera and antigammaglobulin that should be used.

The antihumangammaglobulin used for this study was purchased from a commercial laboratory. The ratio of protein to fluorescein for conjugation used was 20:1 by weight. The mixture of globulin and dye was allowed to sit overnight at 4°C. The mixture was then poured into a small cellophane bag, suspended in a beaker, and the excess dye was removed by dialysis against frequent changes of phosphate buffer solution. The conjugated antihumangammaglobulin is purified by diethylaminoethyl (DEAE) cellulose column chromatography to eliminate nonspecific staining.

Cellulose ion-exchange chromatograph, first developed by Peterson, E.A. and Sober, H.A. (12), made purification of protein solutions possible on a large scale. Recent reports have shown that nonspecific staining from fluorescein labeled antisera, can be effectively eliminated by using the DEAE

column (3, 4, 11, 13). The chromatography method employed is adapted from that described by McDewitt et al (9). Analysis showed that 42% of the total precipitating antibody present in the unlabeled conjugate was eluded by 0.05M PO_4 in Fraction I. In a similar study, as much as 60% of the precipitating antibody was recovered in an 0.06M PO_4 fraction. The remaining antibody was lost, or present in the other fractions, where it was associated with non-specific staining (9).

The chromatography method of purification was found to be more efficient than acetone dried tissue powder adsorption. A greater recovery was obtained with chromatography. There is no problem with bacterial contamination, therefore, large quantities can be prepared at one time. There is also no need for the preparation of a wide variety of specific tissue powder.

Smears of tissue culture containing the antigen and control cells were made on 3X1 inch glass slides. Two circular smears were made per slide and allowed to dry at room temperature for about 40 minutes. The slides were then fixed in acetone at 4°C overnight. Proper fixation of the smear is very important in FA technique. Ideal fixation should inactivate the infectiousness of the pathogen without destroying its antigenic capacity to react with the antibody. Ethanol has been found to destroy the antigenicity of mumps, canine distemper, canine hepatitus and, to a certain extent, influenza virus. Acetone has been found to be the most suitable fixative for the majority of viruses studied.

After fixation, slides were rinsed in phosphate buffer solution, pH 7.5 (PBS 7.5) and dried. Antisera (positive and negative), diluted 1:5 and 1:10 with PBS, were inactivated at 56°C for 30 minutes. Antisera were then applied to the smears with disposable pipettes. Slides were then incubated in a moist chamber at 37°C for 30 minutes. After incubation, antisera were flushed off and slides were washed in PBS for 10 minutes, followed by the application of conjugated antigammaglobulin (diluted 1:5, 1:10 and 1:20 with PBS) to the smears. Slides were then incubated at 37°C for 30 minutes in a moist chamber. After incubation, conjugate were flushed off and slides were washed for 10 minutes with PBS. Slides were then blotted dry and ready for examination under fluorescent microscopy.

A Zeiss fluorescent microscope, with dark field condenser, was used in the laboratory. An exciting filter was placed between light source and condenser to cut off light spectrum greater than 4200Å and a barrier filter was placed between the objective and the eye piece to remove wave-lengths below 4200Å in order to protect the eye from the damaging effect of ultraviolet light.

Microscopes on board the FBM submarines, equipped with proper filters, can be readily adapted for fluorescence microscopy. They already have dark field condensers on board. The main problem on board would be light source. High pressure mercury vapor arcs, Osram HB0200, are used as light source in the laboratory. This is not suitable for use on board a submarine because the mercury bulb constitutes a potential atmospheric contamination hazard. The recently developed, high intensity, quartz iodine lamps (GE, Sylvania) are being considered as a possible substitute.

On examination of the smears under fluorescence microscopy, the 1:10 antisera dilution and 1:10 conjugate dilution appeared to give the best results. The criteria for selecting the proper dilution for the procedure was to choose the greatest dilution that would still yield a clear cut, positive response. The 1:5 dilution of the conjugate gave a great deal of nonspecific fluorescence, while the 1:20 dilution only gave a weak fluorescence. Therefore, the 1:10 dilution of the conjugate was chosen.

	Antisera + Conjugate	Neg. sera + Conjugate	Conjugate
Control cells	O - $\pm$	O - $\pm$	O
Tissue culture with Antigen	++ - +++	O - $\pm$	O

Figure 1 - Results obtained by using 1:10 dilution for both antisera and conjugated antigammaglobulin. Fluorescence graded on a scale of O to ++++.

The in vitro study has demonstrated the validity of the indirect fluorescent antibody technique. It has also established the laboratory technique for the in vivo study.

Ten guinea pigs were inoculated intranasally and intraperitneally with RK culture of para-influenza virus, in an attempt to infect them for the in vivo study. The guinea pigs were bled just prior to inoculation and then on alternate days for 10 days. Smears were made with the leucocytes (White Blood Count (WBC)) separated from the blood samples. IFAT was carried out on the WBC smears for detection of para-influenza virus. Human antisera and antihumangammaglobulin conjugate from the in vitro study were used.

About eight ml of blood were obtained by each ventricular puncture. Five ml of the blood were used for serum preparation. Three ml of the remaining portion were added to two drops of Ethylene Diamine Tetra-acetate (EDTA) (anticoagulant) in a test tube chilled in ice water. Then, 1.5 ml of 5 per cent Dextran in saline were added to the blood, mixed well and allowed to settle for 30-40 minutes. The supernate was removed and centrifuged in a siliconized centrifuge tube for 8 minutes at 900 revolutions per minute (RPM). The supernatant fluid again was removed and the sediment resuspended in phosphate buffer solution (PBS) 7.2. The suspension of WBC, free of serum and antibodies was then ready for smear preparation. Smear preparation, fixation and IFAT followed the same procedure used for the in vitro study.

RESULTS

During the in vivo study, two guinea pigs expired. One died on day two as a result of excessive ether during anesthesia for ventricular puncture. One died on day four as a result of ventricular tear due to the sudden movement of the animal during blood sample collection. No evidence of gross anatomic changes were seen at necropsy.

On examination, using fluorescence microscopy, no fluorescence was seen in the WBC smears prepared from the control and postinoculation blood samples. Sera collected were not tested against the antigen (RK cell culture of para-influenza virus) by IFAT, due to the approaching termination of the off crew period.

Further in vivo study is certainly justified at this stage. Based on the concept of viremia in infection, WBC was chosen as a likely source of antigen. Kohler's (6) observation of influenza virus in leucocytes of experimental animals supports this concept. It is further supported by the report of Liu et al (8) on visualization of distemper virus in the leucocytes of a blood smear of an infected ferret. Polio virus has also been demonstrated by Kovac et al (7) in isolated leucocytes, blood and C.S.F. smears of infected monkeys by IFAT and biological means. It is felt that the absence of positive fluorescent response in the in vivo study is most likely due to the absence of infection or, at any rate, the absence of viremia in the guinea pigs.

SUMMARY

This preliminary investigation has established an indirect fluorescent antibody technique for use in the laboratory; its validity has been demonstrated by in vitro study. Pending future successful demonstration of in vivo studies, the technique, at this stage, can be adapted as a screening test for specific infections on board an FBM. Therefore, it would be a most useful tool in determining whether a small number of viruses are the causative agents of recurrent Upper Respiratory Infections within an FBM crew. It is not to be inferred, however, that we are anywhere near the application of IFAT in the diagnosis of general URI. The immense complexity of the problem can be appreciated when we consider the large number of viruses involved. Hamparian, V.V. et al (5) has defined 53 serotypes of rhino virus in his laboratory. Thirty serotypes of adenovirus are known to affect man (1) not to mention the para-influenza virus, respiratory syncytial virus, and agents that have not as yet been detected. The isolation of these viruses and the preparation of specific antisera would be a tremendous, though not insurmountable, undertaking. However, the IFAT studies of specific viral infections may form the foundation and building block for the eventual application of this technique for rapid diagnosis of URI.

ACKNOWLEDGEMENT

Many thanks to Dr. H. M. S. Watkins, Mrs. T. Stevens, Mr. J. Hresko and the staff of Naval Biological Laboratory for their guidance, technical advice, material support and encouragement, that made this study possible. To the members of USS KAMEHAMEHA Gold Crew, my deep appreciation for their patience and cooperation with their Medical Officer, in spite of the constant harrassment to which he may have seemed to subject them.

REFERENCES

1. Andrews, C.H. Science, 146:1274, 1964.

2. Coons, A.H., Creech, H.J., Jones, R.N. and Berliner, E.J. J. Immun., 45:159, 1942.

3. Curtain, C. C. J. Histochem., 9:484, 1964.

4. Goldstein, G., Slizy, I.S. and Chase, M.W. J. Exper. Med., 114:89, 1961.

5. Hamparian, V.V., Leagus, M.B. and Hilleman, M.R. Proc. Soc. Exp. Bio. & Med., 116:976, 1964.

6. Kohler, H. Zbl. Bakt. Orig. I, 180:140, 1960.

7. Kovac, E., Baratawidjaja, R.K., Walmsley-Hewson, A. and Labzoffsky, N.A. Arch ges. Virusforsch., 14:143, 1963.

8. Liu, C. and Coffin, D.L. Virology, 3:115, 1957.

9. McDevitt, H.O., Peters, J.H., Pollard, L.W., Harter, J.G. and Coons, A.H. J. Immun., 90:634, 1963.

10. McQueen, J.L., Lewis, A.L. and Schneider, N.J. Am. J. Pub. Health, 50:1743, 1960.

11. Peters, J.H., McDevitt, H.O., Pollard, L.W., Harter, J.G. and Coons, A.H. Fed. Proc., 20:17, 1961.

12. Peterson, E.A. and Sober, H.A. J. Am. Chem. Soc., 78:751, 1956.

13. Riggs, J.L., Loh, P.C. and Eveland, W.C. Proc. Soc. Exp. Bio. & Med., 105:655, 1960.

14. Stulberg, C.S. Am. J. Dis. Child., 101:137, 1961.