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OFFICE OF NAVAL RESEARCH

CONTRACT NO. N00014-76-C-0247

WORK UNIT #NR 204-014

TECHNICAL REPORT NO. 6

CULTIVATION OF HEPATITIS VIRUS IN TISSUE CULTURE

BY

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March 1, 1979

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INTRODUCTION

Viral hepatitis remains an important health problem to personnel of military age. Conservative estimates indicate that there are at least 30,000 cases of posttransfusion hepatitis each year as well as an additional large number of cases of hepatitis relating to routes of inoculation other than blood transfusions. Moreover, morbidity reports document at least 3,000 deaths per year from viral hepatitis and recent data suggests that approximately 10 to 14% of patients who have hepatitis B and 20 to 40% of patients who develop non A non B hepatitis will develop chronic liver disease and, of these, a major proportion eventually develop cirrhosis with early death. Furthermore, the morbidity from episodes of acute hepatitis among military personnel is especially significant. Many of our largest epidemics of viral hepatitis have occurred in Naval installations. For example, an outbreak of hepatitis A at the San Diego Naval Base in 1976 resulted in the exposure of 2,000 recruits and clinical illness in approximately 200 enlisted men. Similar episodes have been described at other Naval bases in previous years.

Only in recent years have significant advances been made in understanding the pathogenesis of viral hepatitis. The development of sensitive new serologic techniques has now allowed us to differentiate amongst the forms of viral hepatitis and this in turn has made it possible to consider the development of methods of prophylaxis such as specific viral vaccines. In order to prevent hepatitis by vaccine or gamma globulin, basic research must be accomplished to understand the steps leading to viral replication which in turn result in disease. Only through this understanding will it be possible to successfully cultivate the viruses of hepatitis A, hepatitis B, and non A non B hepatitis. The program established by the Office of Naval Research at the University of California at Los Angeles has been designed to proceed in a stepwise fashion to unravel the complex interactions that lead to the multiplication of hepatitis viruses in human cells. It is our hope that this approach will eventually enable us to develop techniques for the successful propagation of these viruses and the development of effective vaccines or other means of prophylaxis.

METHODS

1. Sources of Potentially Infectious Virus

Our previous reports have documented an approach to the identification of inocula likely to contain hepatitis virus. We have obtained stool specimens from patients in the acute phase of hepatitis A. Electron-microscopic studies have documented that these specimens contain viral particles. Serum specimens were obtained from patients with acute hepatitis B who had been prospectively followed after blood transfusions. Electron-microscopic studies demonstrated the presence of whole virus particles in these serum specimens. The development of materials likely to contain non A non B hepatitis virus required a more difficult undertaking. In order to identify plasma likely to be rich in non A non B hepatitis virus it was necessary to prospectively follow patients after blood transfusion. Fortunately, previous serologic studies had indicated that those patients who received blood units rich in

carcinoembryonic antigen (CEA) which also had elevated serum glutamic pyruvic transaminase (SGPT) levels were likely to eventually develop non A non B hepatitis. Using this information such patients were identified after retrospectively evaluating aliquots of the blood units they received. These patients were prospectively followed and plasma was obtained before, during and after the development of posttransfusion non A non B hepatitis. Utilizing the techniques of affinity chromatography and ultracentrifugation the potentially infectious materials were partially purified and concentrated and subsequently inoculated into experimental tissue culture lines.

2. Preparation of Tissue Culture Systems

Hepatocyte cultures were established using techniques previously developed in this contract program. Cloned hepatocytes were maintained in monolayer culture using a growth medium consisting of RPMI 1640 medium fortified with 20% fetal calf serum, 600 mg/L bactopeptone, 300 mg/L of L-glutamine and 136 mU/L of crystalline insulin. Cultures were grown in a CO_2 incubator at 37°C. Once the monolayer was maintained the growth medium was changed to a maintenance media and the fetal calf serm was reduced to 2%.

Monolayer cultures of small bowel cells were prepared using the techniques described above. The same growth and maintenance media were employed as were the techniques for tissue culture development.

3. Inoculation

Inocula were diluted 1:4 in growth media and 0.3 ml of inoculum was used in each tube or flask. Pelleted, virus-like material which was derived by affinitiy chromatography and subsequent ultracentrifugation was resuspended in, and dialyzed in, three changes of normal saline. 0.3 ml of each inoculum was then placed in six tissue culture tubes or flasks. Control material consisted of sera prepared in a similar manner using the same techniques but obtained from race, age, and sex matched patients who did not have hepatitis. For the hepatitis A studies stool specimens which had been partially purified were utilized from similar matched controls.

RESULTS

Initial studies confirmed the presence of virus-like particles in the hepatitis A and hepatitis B inocula. These particles have a configuration and appearance previously documented for hepatitis A and hepatitis B viruses. Furthermore, specific antibody directed against these agents produced agglutination of virus particles when examined under the electron-microscope. Electron-microscopic studies of the non A non B preparations did not reveal particles thought to represent viruses. Others have also failed to demonstrate electron-microscopic evidence of virus particles in sera obtained from non A non B patients even though such sera had been shown to produce hepatitis. However, because of the question of the infectivity of this material we also utilized plasma obtained from blood units which later were shown to cause hepatitis in the recipients of those units. Thus, in the ongoing study described in previous reports we had frozen away aliquots of blood units transfused to our patients. When such patients then developed non A non B hepatitis we were able not only to obtain specimens from the hepatitis patients

but also to go back into our freezers and remove aliquots of the blood units which actually caused non A non B hepatitis. These aliquots were examined under the electron-microscope and also failed to show evidence of virus-like particles. Nevertheless, since we knew that they had resulted in hepatitis in their recipients they were used for tissue culture inoculation. Since many of our recipients received three to five blood units it often was necessary to inoculate tissue culture with aliquots of units in which we were not certain which unit actually produced non A non B hepatitis but in which we did know that at least one of the inoculated units must have produced the disease.

Thus far, none of the inocula have resulted in the development of cytopathic change in the tissue culture systems studied. However, when an interference assay is used, similar to that used for the detection of rubella virus, evidence of an interfering agent has sometimes been documented in those culture systems inoculated with material containing hepatitis A virus. Testing the ability of specific antibody to inhibit this interference has thus far failed to confirm that an interfering viral agent has been cultivated. However, the data is suggestive that this may be occurring. Interference tests when applied to the hepatitis B inoculated material or the non A non B inoculated material have not resulted in evidence of viral replication.

DISCUSSION

Scientists have struggled for four decades in an attempt to cultivate the hepatitis virus. Clearly this is not, and will not be, an easy task. However, if effective vaccines are to be developed it is likely that the best vaccine will come only if virus is successfully cultivated in tissue culture systems. To accomplish this, scientists must understand the unique aspects of these unusual viruses. The first step in understanding the interaction between virus and host cell must be in assessing the ability of viruses to establish infection in various tissue culture systems. Thus, this program was designed to meticulously develop several tissue culture systems likely to support hepatitis viruses and then to assess the steps in viral infection including attachment, entrance into the cell, replication of viral nucleoprotein and eventually immune cytolysis of the cell system if such is to develop. To accomplish this, we have successfully developed unique tissue culture systems and have reproducibly shown that these cell lines can be maintained not only for short periods of time but also for long time intervals. Recently, we have developed materials likely to be highly infectious and proven to have caused hepatitis in human recipients. Our current studies are aimed at utilizing these unique reagents in assessing the mechanisms by which hepatitis viruses may replicate in human cells.

FUTURE PLANS

Based on current data, one step in understanding the ability of hepatitis virus to infect human cells may be in the development of appropriate interfering assays in the search for interferon-like substances which may be produced by hepatitis viruses. A major insight into the mechanisms of virus-cell interaction may result. In addition, in order to assay non A non B hepatitis virus, future studies must concentrate on the development of a

serologic assay system. Future studies should be aimed at developing a sensitive radioimmunoassay or enzyme immunoassay for the *in vitro* detection of non A non B hepatitis virus.

PERTINENT PUBLICATIONS

1. Gitnick GL, Stone OW, Brezina ML: Transfusion transmitted carcinoembryonic antigen, serum glutamic pyruvic transaminase and hepatitis. (In Submission)

2. Gitnick GL, Brezina ML, Mullen RD: Application of alanine aminotransferase, carcinoembryonic antigen and cholyl glycine levels to the prevention and evaluation of acute and chronic hepatitis. In: Viral Hepatitis , Vyas GN, Cohen SN, and Schmid R, Eds, Franklin Institute Press, Philadelphia, PA, 1978, pp 431-438.

Unclassified

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER: 6	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle): (6) CULTIVATION OF HEPATITIS VIRUS IN TISSUE CULTURE.		5. TYPE OF REPORT & PERIOD COVERED: Annual Report March 1, 1978 to February 28, 1979
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s): (10) G.L. Gitnick, ~~M.D.~~		8. CONTRACT OR GRANT NUMBER(s): (15) N00014-76-C-0247
9. PERFORMING ORGANIZATION NAME AND ADDRESS: University of California at Los Angeles, 10833 Le Conte Avenue, Los Angeles, California 90024		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: Office of Naval Research Work Unit #NR 204-014
11. CONTROLLING OFFICE NAME AND ADDRESS: Office of Naval Research, 1030 East Green Street, Pasadena, CA 91106		12. REPORT DATE: March 1, 1979
		13. NUMBER OF PAGES: 4
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office): (14) TR-6		15. SECURITY CLASS. (of this report): Unclassified
(12) 6 p.		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

This document has been approved for public release: its distribution is unlimited.

(11) 1 Mar 79

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

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18. SUPPLEMENTARY NOTES

(9) Technical rept. 1 Mar 78-28 Feb 79,

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Hepatitis Virus
Hepatitis B Antigen
Tissue Culture
Cultivation of Hepatitis Virus
Hepatocyte cultures
Hepatitis Vaccine
Non A Non B Hepatitis

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Viral hepatitis is an illness of increasing importance to persons of military age. Although hepatitis A is not associated with chronic liver disease it is associated with important morbidity. Hepatitis B leads to chronic liver disease in 10 to 14% of patients while non A non B hepatitis produces chronic liver disease in 20 to 40% of patients. Because of these important sequelae of viral hepatitis it is important that efforts be undertaken to understand the interactions between the virus and human host cells.

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Unclassified 408 775

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Through this knowledge, eventually techniques may be developed leading to safe and effective live viral vaccines. To accomplish these ends we have developed tissue culture systems utilizing human hepatocytes and others composed of small bowel cells for the eventual cultivation of hepatitis viruses. Recently we have been successful in developing reagents likely to contain infectious virus. Thus, stool specimens from hepatitis A patients have been obtained and purified. Serum specimens from hepatitis B patients have been obtained and are known to contain whole virus particles. Through studies of posttransfusion hepatitis, infectious plasma known to produce non A non B hepatitis has been identified as has convalescent sera from such patients. Tissue culture studies have been undertaken for the cultivation of hepatitis viruses. Evidence of cytopathic change has not been seen with any of the viral isolates of any of the viral material. However, with hepatitis A, a possible interfering agent has been suggested but definitive evidence in this regard is not available. Work is continuing aimed at further efforts at defining virus host cell interactions in order to stimulate intracellular viral replication.