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CULTIVATION OF HEPATITIS VIRUS IN TISSUE CULTURE.(U)
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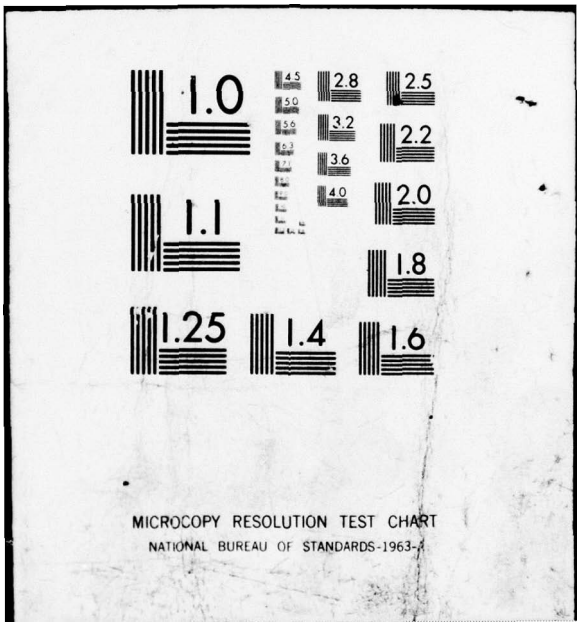
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TECHNICAL REPORT NO. 5

CULTIVATION OF HEPATITIS VIRUS IN TISSUE CULTURE

BY

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INTRODUCTION

The forms of acute viral hepatitis include hepatitis A, hepatitis B, and the most common form of hepatitis has been designated non A non B hepatitis. Hepatitis A generally carries with it a short incubation period and is usually transmitted by fecal or oral contamination. Hepatitis B is the least common form of viral hepatitis, is usually transmitted by contaminated blood products or needles but may be transmitted by the fecal-oral route and often has a longer incubation period. The most common form of hepatitis, non A non B hepatitis may represent viral disease of one or more etiologies. It has an incubation period varying from 6 to 24 weeks, may be transmitted either by blood products, needles or the fecal-oral route and is clinically indistinguishable from the other forms of hepatitis. This common form of hepatitis requires additional scientific evaluation but this has been hampered by the absence of serologic assays for its detection.

The average period of disability for any of the forms of acute viral hepatitis is approximately 5 weeks. In addition, between 1 and 3% of patients will die with acute viral hepatitis due to the development of fulminant fatal hepatitis. Approximately 14% of patients will develop either chronic persistent or chronic active hepatitis. Of those who develop chronic active hepatitis most will develop post necrotic cirrhosis. Because of the significant morbidity and eventual mortality associated with these illnesses it is important that efforts be directed at the cultivation of hepatitis viruses and eventually at the development of effective and safe vaccines.

Recently, Watanabe, et al,[6] developed a new technique for the cultivation of hepatitis B virus. They utilized oval hepatocytes which flow freely in the media after liver explants are planted. These hepatocytes maintain the function of normal liver cells including the production of albumin and alpha-1-fetoprotein. They are intermediate in appearance between an epithelial cell and a fibroblast and have an oval shape. On initial passage, immunofluorescent studies demonstrated the presence of hepatitis B surface antigen in these hepatocytes. In our laboratory we have previously been unable to demonstrate cytopathic change or increase in antigen titer using standard assay procedures. However, Watanabe uniquely applied immunofluorescence to his assay system and was able to demonstrate persistence of viral antigen. Furthermore, he was then able to subculture oval cells up to 14 passages and demonstrate the continued presence of specific immunofluorescence with each subpassage. We have recently undertaken similar studies.

During the past year we have developed methods for the detection of blood units likely to contain the agent of non A non B hepatitis. We have recently undertaken the application of these new methods to the needs of this hepatitis vaccine development program.

METHODS

1. Sources of Non A Non B Virus and Antigen

Patients at the University of California, Center for the Health Sciences, Los Angeles, were entered into a prospective study of PTH. Criteria for inclusion were the absence of a history of jaundice, hepatitis, clinical or biochemical evidence of liver disease, blood transfusion within the previous nine months, hepatotoxins, or a life expectancy of less than nine months. A prerequisite for enrollment was a normal SGPT and CEA level in the patients' blood samples prior to transfusion. After transfusion, blood specimens were obtained every two weeks for

ten months. These samples were tested for HBsAg (Ausria II, Abbott Laboratories, North Chicago, Illinois), SGPT (Beckman Automated, kinetic method), and CEA (CEA Roche, Hoffmann-La Roche, Inc., Nutley, New Jersey). The diagnosis of PTH was made if the patient was found to have two SGPT levels greater than five times the upper limit of normal drawn five to 21 days apart. The episode was termed non A non B PTH if the patients were negative for HBsAg during the acute phase and failed to develop either anti-HBs or anti-HA during convalescence.

The donors of blood units with elevated CEA and SGPT levels from the past study, implicated in the transmission of non A non B PTH, were contacted Blood samples were drawn in EDTA for CEA testing and as serum for HBsAg, anti-HBc and SGPT testing. Those donors with persistently abnormal CEA and SGPT were asked to undergo a single unit plasmaphoresis. Transfusion recipients who developed non A non B PTH during this study, who had persistently abnormal SGPT (greater than 45 I.U.) with or without CEA elevation were asked to undergo a one unit plasmaphoresis. All such plasma was coded and stored at -70°C.

All outdated plasma from the UCLA Blood Bank was provided to our group. All units were screened for SGPT. All HBsAg negative units with SGPT levels greater than 50 I.U. were assayed for CEA. Units with SGPT levels greater than 50 I.U. and CEA levels greater than 4.9 ng/ml were coded for use as a source of antigen. In addition, HBsAg negative units with SGPT levels greater than 100 I.U. were coded and stored at -70°C as an alternative source of antigen regardless of CEA levels.

Blood specimens (10 ml) were obtained in EDTA at the time of voluntary blood donations for future CEA testing. An additional blood specimen was obtained for SGPT testing from all donors presenting to the UCLA Blood Donor Center during a 90 day period in each calendar year. Those specimens were stored at -70°C and retrospectively tested over a two week period following transfusion. During that period all recipients of blood from the UCLA Blood Donor Center were evaluated. Those who lacked evidence of malignancy, who were receiving no hepatotoxic drugs, who had normal SGPT levels prior to transfusion, who denied any history of prior liver disease and who were willing to participate in a prospective study were enlisted. All patients receiving one or more blood units with an elevated CEA level were matched with patients of the same race, age, sex and smoking history, who had a similar disease state and who had received all volunteer blood in which no units had CEA elevation. Similarly, all patients receiving a blood unit with an SGPT level over 50 I.U. were matched in the same way with patients receiving blood units without SGPT elevation. Patients were then followed at two week intervals for 10 months. Blood specimens were tested for SGPT and for CEA.

Donors with both SGPT and CEA elevation or with SGPT elevation alone, implicated in the transmission of non A non B PTH in this prospective study were reassayed for SGPT and CEA. If persistently abnormal, they were asked to undergo a one unit plasmaphoresis.

Patients developing non A non B PTH during prospective study had 60 ml of blood drawn weekly (10 ml in EDTA) for the duration of their acute illness. If such patients had persistent SGPT elevation for greater than six months, they were asked to undergo a one unit plasmaphoresis.

All serum and plasma were stored at -70°C pending further processing. Thus, the potential sources of non A non B PTH virus/antigen available for examination included:

1) Donors with an elevated CEA and SGPT implicated in the transmission of non A non B PTH.

2) Donors with an elevated SGPT but with normal CEA implicated in the transmission of non A non B PTH associated with a normal CEA level during the acute phase of illness.

3) Recipients of such blood with acute and chronic non A non B PTH.

4) Outdated bank plasma with CEA elevation and/or SGPT elevation

2. Sources of Non A Non B PTH Antibody

Patients with non A non B PTH identified by the prospective studies were followed for resolution of their hepatitis (return of the SGPT level to normal). After six weeks to six months convalescence from non A non B PTH, patients were asked to undergo one-unit plasmaphoresis at the UCLA Blood Donor Center. Plasma was coded and stored at -20°C. These samples will provide the source of non A non B antibody.

3. Controls

Age and sex matched donor controls and outdated plasma controls with normal SGPT and CEA levels were coded and stored at -20°C. Age, sex and disease state matched hospital controls who were either not transfused or transfused with blood without an elevated SGPT or CEA level, were part of the above described prospective study. Their serum samples, stored at -70°C are available for controlled comparison.

4. Tissue Culture

Serum samples likely to contain non A non B PTH virus (see 1 above) and virus-like material derived from affinity chromatography and ultracentrifugation will be inoculated into tissue culture cell lines available and in use in this laboratory.

a. Hepatocyte Cultures

Hepatocyte cultures will be established using explants of adult and fetal liver tissues. Explants will be cultures in growth medium consisting of RPMI 1640 medium fortified with 20% fetal calf serum, 600 mgm/L bactopeptone, 300 mgm/L of L-glutamine and 136 Mu/L of crystalline insulin. Cultures will be grown in a CO_2 incubator at 37°C and the liberated floating cells will be collected on the 8th day. They will then be centrifuged in Ficol-Corary (24:10) for 30 minutes at 1500 rpm. Oval cells will be distributed under the lymphocyte layer. The oval cell fraction will be removed and will be used as a substrate for subsequent studies.

Oval cell suspension cultures will be used to evaluate the report of Watanabe, et al,[6,7] regarding the reproducible subpassage of hepatitis B virus in oval cells. The inocula described above will be used for these studies. When and if confirmed, additional hepatocyte culture experiments will be undertaken. The oval cell rich fraction will be planted as an explant culture in falcon plastic dishes. Once an adequate monolayer has been developed using an explant technique of a previously isolated oval cell rich fraction, the growth medium described above will be altered in that the concentration of fetal calf serum will be reduced to 2% for maintenance.

In addition to the employment of monolayer cultures, oval cells will be grown in co-cultivation with human diploid lung cells, Hep-2 cells and HeLa cells. For each of these studies, one volume of hepatocyte cells containing approximately 10^5 viable oval cells per ml will be planted in a specially devised falcon plastic dish separated by a small elevated line which passes through the middle of the dish. On the other side of the dish, cells such as HeLa, Hep-2 or human diploid lung cells will be planted in the same concentrations. Each monolayer will be grown in the same dish and will be bathed by the same growth and maintenance media. The hepatocytes will be inoculated with each of the inocula listed below in the hope that viruses may replicate within the hepatocytes and may then pass to the other tissues where they may more easily demonstrate cytopathic change or positive immunofluorescence. Because of differences in growth rates, it may be necessary either to plant on different days or to alter the frequency of fluid changes. Control cultures will include cultures of tissue substrates only and each of the standard tissue cultures separately. Cultures will be examined daily for evidence of cytopathic change and a portion of each group of cultures will be examined every four days for specific immunofluorescence. Any cultures showing cytopathic change or positive immunofluorescence will be serially passaged to establish a viral pool of sufficient size for further studies. If control cultures reveal the presence of adventitious agents, such as Simian virus (SV-40), that line of tissue will be discarded and fresh cultures will be reinoculated with the previous passage level. Tissue culture aliquots from each passage level will be stored in liquid nitrogen for future reference and if necessary for replanting. Immunofluorescence, electron microscopy and immunoelectron microscopy will be used to search for evidence of viral growth. Indirect immunofluorescence will be employed in selected cultures using the sera of patients with high levels of hepatitis B surface antibody as determined by the hepatitis B surface antibody radioimmunoassay. Human antibody to hepatitis B antigen and goat antibody to hepatitis B antigen are also available for use. Selected cultures will be examined by electron microscopy and by immunoelectron microscopy for evidence of viral replication. Cultures will be removed at eight day intervals for electron microscopic studies. If any agent is detected, or if evidence of viral growth is demonstrated, attempts will be undertaken to neutralize the agents using convalescent hepatitis sera. If feasible, the virus will be adapted to and serially passaged in Riff free chick embryo culture in order to produce a viral vaccine appropriate for federal licensure.

b. Viral Inocula

Watanabe has used plasma from patients with fulminant hepatitis B for his successful studies of hepatitis B surface antigen cultivation. We have available to us a unique source of sera from such patients. This large medical center in 1975 and 1976 cared for more patients with fulminant hepatitis than any other medical center in the United States and entered more patients in the National Fulminant Hepatic Failure Collaborative Program. We have available to us sera from such patients rich in hepatitis B surface antigen and rich in Dane particles and we propose to use representative plasma for these viral inoculation studies. In addition, we have collected sera from patients found to have hepatitis A. Sera has been collected at seven day intervals from patients who initially lacked hepatitis A antibody but subsequently, after exposure, developed acute viral hepatitis and eventually developed high levels of anti-hepatitis A. Such serial plasm specimens will be available for these viral cultivation studies. Finally, we also have available aliquots from blood units which produce non A non B hepatitis on prospective follow-up of recipients as previously described.

c. Non A Non B Culture Inoculation

Serum inocula will be diluted 1:4 in growth medium and a 0.3 ml of inoculum will be used per tube or flask. Pelleted, virus-like material derived from affinity chromatography and ultracentrifugation will be resuspended in, and dialyzed in, three changes of normal saline. 0.3 ml of this material will be inoculated into each of four tissue culture tubes and flasks. Control sera, similarly diluted and similarly processed by column chromatography and ultracentrifugation will be used as control inocula. Viral replication will be assessed by daily microscopic observation and, after one to two weeks, by electron microscopy of fixed monolayers.

RESULTS

In a pilot program, we evaluated means of preventing non A non B hepatitis. Prior to transfusion, patients were identified who lacked evidence of malignancy or chronic liver disease, who had no history of blood transfusions in the prior 12 months, who had not been administered hepatotoxic drugs, and who were likely to survive for a ten month period of follow-up. Blood units were obtained from both commercial and voluntary donors. Aliquots from the transfused units were obtained in ethylenediaminotetraacetic acid (EDTA), were stored at -70°C and were retrospectively screened using the CEA radioimmunoassay of Hansen, et al (CEA Roche, Hoffmann-La Roche, Inc., Nutley, New Jersey). We had previously observed the frequent development of this fetal antigen during PTH. After identification of CEA positive blood units, the recipients of these units were sought. Recipients of CEA positive blood were selected on the basis of having received units containing CEA in concentrations greater than 2.5 ng/ml and subsequently were divided into those with CEA levels between 2.5 and 4.9 ng/ml and those above 4.9 ng/ml. All of the other blood units transfused to these patients as well as all blood units transfused to patients in the control groups were then screened for the presence of CEA.

For each recipient of CEA positive blood, two control subjects were randomly selected. One control subject was a recipient of the same number of blood units, all of which were negative for CEA. These controls were matched for sex, age, race and the total number of blood units, commercial and otherwise received. The other control subject was a patient without malignant disease of the same race, age, and sex, who was hospitalized but who received no blood transfusions.

All blood units were free of HBsAg as determined by RIA (Ausria II, Abbott Laboratories, North Chicago, Illinois). A pre-transfusion specimen was obtained in EDTA and 30 ml of plasma was removed from every blood unit administered to each study patient and also placed in EDTA for future CEA testing. In calculating the CEA results, the dilutional effect of the acid citrate dextrose anticoagulant of the blood was taken into account. All patients and controls were followed for ten months. Plasma specimens in EDTA and serum specimens were obtained prior to transfusion and at two week intervals for the ten months of follow-up. Serum specimens were tested for HBsAg, anti-HBs (Ausab, Abbott Laboratories, North Chicago, Illinois), bilirubin, alanine amino transferase (SGPT) and antibody to hepatitis A (RIA, Abbott Laboratories, North Chicago, Illinois).

Hepatitis was defined as an SGPT greater than five times the upper limit of laboratory normal (upper limit normal = 45 I.U.) on one occasion and at least two times the upper limit of laboratory normal on a separate occasion at least five days apart in the absence of any other likely cause of liver disease. Type B viral

hepatitis was defined as the development of HBsAg during the acute phase of hepatitis as described above or the development of anti-HBs during convalescence.

The initial screening of 486 HBsAg negative units revealed 86 (18%) with CEA concentration over 2.5 ng/ml, 50 (10%) of these units had CEA concentrations over 4.9 ng/ml. Among the 86 recipients of this blood with abnormally elevated CEA, 29 (34%) developed non A non B PTH. All 29 of these patients had transient elevation of their CEA levels to above 2.5 ng/ml during the acute phase of their illness. Using the higher cut off for abnormal CEA of 4.9 ng/ml. we found that 23 (46%) of these recipients developed non A non B PTH with transient CEA elevation. Of the 86 recipeints in the control group, all of whom received blood with CEA levels below 2.5 ng/ml, 8 (9%) developed non A non B PTH. In none of these eight cases was there an elevation of the recipients' CEA level to above 2.5 ng/ml during their hepatitis. Among the non-transfused hospital controls, there were no cases of hepatitis.

Because blood donors whose blood units transmit hepatitis may themselves have asymptomatic liver disease, the transfused blood was also screened for SGPT. Of 16 recipients of blood units with SGPT levels over 50 I.U. and CEA levels over 4.9 ng/ml, all developed hepatitis while ten recipients of blood with elevated SGPT and normal CEA levels failed to develop hepatitis.

DISCUSSION

Viral hepatitis remains a critical national health hazard. By recent estimates there are over 30,000 cases of post transfusion hepatitis (PTH) each year resulting in approximately 3,000 deaths and additional unestimated morbidity from chronic hepatitis. Historically two forms of viral hepatitis were distinguished by their lack of cross-immunity in human transmission experiments and other epidemiological differences.[2-4] Infectious hepatitis (type A viral hepatitis) was found to have a short incubation period of from two to six weeks, and was transmitted by contaminated food, water and by the fecal-oral route. In contrast, homologous serum jaundice (type B viral hepatitis) was found to have a longer incubation period of three weeks to six months and was mainly transmitted parenterally by contaminated serum. Later, detailed epidemiological studies of PTH (the "homologous serum jaundice" described by McCallum, et al, Paul, et al, and Neefe, et al) by Allen and Sayman, revealed a skewed unimodal distribution of incubation periods, not the bimodal curve one would anticipate if PTH were the result of a combination of types A and B hepatitis. However, advancement in this area of research was not possible until the discovery of the Australia antigen by Blumberg, et al, and its eventual identification as the hepatitis B surface antigen (HBsAg). With the development of successively more sensitive tests for the detection of HBsAg and other hepatitis B viral markers, particularly antibody to the hepatitis B core antigen (anti-HBc), it became evident that 50%-70% of PTH was not a consequence of hepatitis B virus infection. Furthermore, epidemiological evidence indicated that hepatitis A was also not responsible and objective serology excluded the Epstein-Barr virus and cytomegalovirus as etiologic agents. The discovery by Feinstone, et al, of the hepatitis A antigen in the stool of experimentally infected humans by immune electron microscopy and the resulting objective serological test for hepatitis A finally provided firm serological evidence that non B PTH was also not type A viral hepatitis. These data have been amply confirmed at several centers using the newer assays for antibody to the hepatitis A antigen, immune adherence hemagglutination and radioimmunoassay (RIA).

Several studies have assessed the reduction in PTH resulting from the use of sensitive methods for HBsAg screening, the use of volunteer as opposed to commercial

blood donors and the use of prophylactic gammaglobulin to prevent PTH. Testing for HBsAg by RIA or a test of equivalent sensitivity has resulted in at least 50% and possibly a 90% reduction in type B PTH but has had no impact on non A non B PTH. The substitution of volunteer blood donors resulted in a marked decrease in both types B and non A non B PTH. But even using both a volunteer blood supply and RIA testing for HBsAg, there was left a 5%-15% incidence of non A non B PTH among transfused patients. While the prophylactic use of human immune serum globulin has reduced the icteric, overt, acute phase of PTH, the overall incidence has remained unchanged. The morbidity of this disease is increased by the frequent progression of acute non A non B PTH to chronic hepatitis. The reported incidence of chronic hepatitis following non A non B disease varies from 23% in the study of Knodell, et al, in which some patients received prophylactic gammaglobulin to 70% in the study of Koretz, et al, in which 85% of blood transfused was commercial and in which gammaglobulin was not used. The report by Knodell, et al, that prophylactic human immune serum globulin may reduce this progression to chronicity needs to be confirmed. But even with gammaglobulin prophylaxis, substantial progression to chronicity remains. Many of these patients had biopsy features of chronic hepatitis and cirrhosis, diseases which in other instances have been shown to progress relentlessly.

The close epidemiological and clinical similarity between type B hepatitis and non A non B hepatitis makes it likely that the latter is also associated with a chronic viremia or chronic carrier state. Evidence of such a chronic carrier state was reported by Hoofnagle, et al, who found, in reanalyzing the human transmission experiments by Murray, et al, that three donors implicated in the transmission of non A non B hepatitis transmitted the disease on separate occasions as much as 385 days apart. These data suggest that the reservoir for non A non B PTH is found in the liver and circulation of chronic carriers making carrier blood an appropriate target for investigations which might result in the identification and isolation of the presumed viral agent or agents of this disease. However, there is no reason to believe that the viremia of non A non B PTH will have any of the unique features of hepatitis B which made the latter assayble by relatively insensitive means. Thus, the milligram quantities of excess HBsAg circulating in chronic hepatitis B are unique in virology. In contrast, the viremia of other human diseases comprise quantities of whole virus not assayable by even the most sensitive methods, such as RIA, in unconcentrated serum specimens. Instead the isolation of non A non B hepatitis antigen from blood would be expected to require biophysical and immunological concentration as was required to isolate the core of the complete hepatitis B virus from plasma.

Reproducible and reliable techniques were developed both for short and long term maintenance of hepatocytes in monolayer tissue cultures. These cultures were free of fibroblastic cells and retained epithelial characteristics. They were maintained for periods in excess of ten weeks. The development of this substrate constituted the initial phase of a program with the eventual goal being the development of a viral vaccine for hepatitis B and for other forms of hepatitis. The second phase of this program undertook to cultivate hepatitis virus in hepatocyte tissue cultures. Initial studies demonstrated electron-microscopic evidence of hepatitis B antigen within tissue culture cells during the first week after inoculation. Serological studies demonstrated evidence of hepatitis B surface antigen in pour off fluids for periods ranging from four to ten days after inoculation. Recent studies aimed at the long term maintenance of infected oval cell cultures have not yet revealed direct evidence of viral replication in oval cell suspension cultures. However, in related studies it has been found that blood

units with elevated SGPT and CEA levels usually transmit hepatitis. Aliquots from units known to have transmitted hepatitis have been obtained and these will be used as inoculum for new tissue culture studies.

Epidemiological evidence suggest that there may be more than one virus of non A non B PTH. Thus, Mosley, et al, reported three patients with four distinct bouts of hepatitis. In each case two of these four bouts could not be related to either the hepatitis A or B viruses by sensitive serological assays. Most of these attacks occurred in the context of intravenous drug abuse. Independent evidence to support the existence of more than one non A non B hepatitis virus was developed by our group which showed two distinctive serological patterns in this disease. In one form of non A non B PTH, implicated donor blood was found to have elevated carcinoembryonic antigen (CEA) levels above 4.9 ng/ml. The transfusion of such blood resulted in a form of non A non B PTH which was associated with abnormally elevated CEA levels in the recipient during the acute phase of the disease. When other patients, transfused with blood containing normal levels of CEA, developed non A non B PTH it was invariably without CEA elevation. There was no "cross-over" between these two groups, that is blood with a normal CEA level never resulted in hepatitis associated with an acutely increased CEA level, while blood with an elevated CEA level never resulted in PTH with normal CEA levels during the acute illness.

Research in non A non B hepatitis is currently at a point similar to that of hepatitis B research before the discovery and understanding of the Australia antigen (HBsAg) or hepatitis A research before the detection of the hepatitis A antigen. The disease has been defined epidemiologically, clinically and by the exclusion of other etiologies but further progress in this field awaits the identification and isolation of the responsible agents or their antigenic markers. The overall problem can be reduced to that of identifying sources of non A non B hepatitis antigens and antibodies followed by isolation in sufficient quantities to allow for specific serologic assays.

CONCLUSIONS

In summary, in our earlier work we were able to develop reproducible and reliable techniques for the long term maintenance of adult and fetal hepatocytes in monolayer cultures. These hepatocytes were shown to produce albumin and subsequently to produce alpha-1-fetoprotein. In these studies, persistence of hepatitis B surface antigen could be demonstrated for a period of about ten days but evidence of viral replication was not obtained. Recently, a new hepatocyte cultivation technique employing the use of oval cells in suspension cultures of liver explants suggested the persistence of hepatitis B antigen when studied by immunofluorescence. Thus far in our studies only non-specific immunofluorescence has developed and evidence of actual viral replication is lacking. However, recent evidence has shown that blood units which contain elevated SGPT levels and elevated CEA levels have produced hepatitis in each of 16 recipients of such units. Aliquots from these units have been frozen away for testing in tissue culture systems to determine if the systems will support hepatitis virus growth.

ACCOMPLISHMENTS TO DATE

This laboratory has developed unique techniques for the cultivation of hepatocytes in monolayer culture. We have methodically moved through a series of developmental phases with the eventual goal being the development of liver attenuated hepatitis virus vaccines. Initially, techniques for the successful cultivation of fetal hepatocytes and subsequently techniques for the cultivation of adult hepatocytes were developed. We documented the first evidence of long-term cultivation of

fetal hepatocytes for periods of several weeks. These long term hepatocyte cultures were shown to produce albumin and alpha-1-fetoglobulin. Studies identifying the conditions necessary for viral attachment, infection and replication were undertaken. Optimal conditions for growth of hepatocytes including temperature, CO_2 concentration and serum concentrations were determined.

Once the technology for the maintenance of long-term hepatocyte cultures was established, attempts were made to use these cultures as a substrate for the growth of hepatitis viruses. Hepatitis B infective material including sera rich in whole virus particles were inoculated into tissue culture under a variety of conditions and evidence of viral persistence for a period of about ten days was established. However, definitive evidence of viral replication was lacking. In addition, filtrates of stools of patients with hepatitis A were used as inocula for hepatocyte cultures but these failed to demonstrate cytopathic change. Liver biopsies obtained from patients with hepatitis B surface antigen positive chronic aggressive hepatitis were grown in monolayer culture and in co-cultivation both with normal hepatocytes and with other standard tissue culture substrates. These, however, failed to demonstrate evidence of viral replication. The adaptation of "slow virus techniques" including co-cultivation, feeder layer cultures, and long-term maintenance cultures also failed to yield evidence of viral growth as did the use of "complexes" of virus, antibody and complement.

We have undertaken to develop systems for the production of tissue culture substrate thought to be likely to support hepatitis virus growth. We have evaluated these new tissue culture systems in carefully controlled studies. The following describes the results of this program over the past six months.

Hepatocyte cultures were established using explants of adult and fetal liver tissue. Explants were grown in a CO_2 incubator at $37^{o}C$ and the liberated floating cells were collected on the eighth day. They were centrifuged for 30 minutes at 1800 rpm. The oval cell fraction was removed and was used for a substrate for subsequent studies. In these studies the oval cell fraction was planted as an explant culture in Falcon plastic dishes using the technique described by Watanabe.[6]

Cultures were inoculated with plasma from patients with fulminant hepatitis B rich in hepatitis B surface antigen. The cultures were then examined daily for the development of cytopathic change and a portion of each group of cultures was examined for specific immunofluorescence. Cells were also embedded in Epon and examined for electron microscopic evidence of viral particles.

Because blood donors whose blood units transmit hepatitis may themselves have asymptomatic liver disease, it thus, would be a valuable resource for cultivation of non A non B hepatitis virus. Transfused blood was screened for SGPT and for CEA the latter having been shown to develop during the course of acute hepatitis. The units of blood containing CEA and SGPT elevations were identified and have been inoculated into hepatocyte tissue culture systems.

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

Since hepatitis virus has the propensity in vivo to infect human hepatocytes it seemed appropriate to undertake attempts at hepatitis virus cultivation using hepatocyte cultures. Early studies led to technology for the cultivation of fetal hepatocytes with the exclusion of fibroblastic cells. Similar approaches were used to cultivate adult hepatocytes and most recently these studies have demonstrated the successful long-term cultivation of adult hepatocytes for periods in excess of 10 weeks. The conditions for growth

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including temperature, serum content and appropriate antibiotics have been delineated and the methods ~~developed~~ are now reproducible.

In the past twelve months, attempts ~~have been~~ were made to use hepatocyte cultures as a substrate for hepatitis B and hepatitis A cultivation. The inocula used have been hepatitis B antigen positive sera known to be rich in Dane particles, filtrates of stools from patients with hepatitis A and liver biopsies obtained from patients with hepatitis B surface antigen positive chronic aggressive hepatitis. Inoculation of cultures with Dane particle-rich hepatitis B sera has not resulted in cytopathogenic change (CPE) in monolayer cultures. Efforts have been devoted at attempts at cultivation of hepatitis B virus by using "slow virus techniques". By means of co-cultivation, mixed monolayers of normal hepatocytes were co-cultivated with hepatocytes obtained from liver biopsies of patients with hepatitis B surface antigen positive chronic aggressive hepatitis. The cell layers which resulted were maintained and were sub-cultured but did not yield evidence of either CPE or evidence of multiplication of hepatitis B surface antigen. Recently inoculated suspension oval cell cultures have demonstrated positive immunofluorescence for HBsAg suggesting possible early viral growth.