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TECHNICAL REPORT NO. 4

CULTIVATION OF HEPATITIS VIRUS IN TISSUE CULTURE

BY

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Introduction

During the past year, studies have revealed that using a new culture technique, hepatitis B surface antigen can be detected by immunofluorescence of multiply passaged hepatocyte oval cells. This approach to virus cultivation suggests that hepatitis B virus may replicate in a tissue culture system and may be subpassaged. If so, this data provides a unique approach to virus cultivation and the possible eventual development of a live attenuated virus vaccine for hepatitis B. This same approach may be applied to hepatitis A and to non A non B hepatitis.

Over the past decade a variety of approaches to virus cultivation have been undertaken with the eventual aim of cultivating hepatitis virus but each has met with failure. During this time period, new serologic techniques have successfully defined at least three common kinds of viral hepatitis. These include hepatitis A which is generally associated with endemic hepatitis in the community usually transmitted by the fecal-oral route, hepatitis B which may be transmitted either by the systemic route such as by blood transfusions or through the use of contaminated needles or may be transmitted by non-parenteral routes, and non A non B hepatitis which may represent one or more forms of viral hepatitis whose etiology remains unclear. With the development of highly specific and precise techniques for the serologic identification of hepatitis viruses, increased efforts have been devoted to the eventual cultivation of these agents and to the development of a live viral vaccine. Because cultivation was not successful after a number of attempts, an alternate route for the development of an inactivated hepatitis B vaccine was adopted. This led to the preparation of highly purified hepatitis B surface antigen particles obtained from human serum, subsequently inactivated and prepared as an experimental vaccine. It is likely that this inactivated vaccine will be available in the coming decade. However, other viral vaccines which are inactivated rather than live and attenuated usually are not highly immunogenic nor do they provide long lasting protective antibody. Furthermore, a vaccine prepared, even in a highly purified form, from human serum may have important problems with regard to safety. Accordingly, it is appropriate to undertake studies aimed at the cultivation and attenuation of hepatitis viruses if technology indicates that such a goal is likely to be attained.

Recently, Watanabe, et al, have developed a new technique for the cultivation of hepatitis B virus. This same technique was developed along a parallel course in our laboratory. Both groups have 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 demonstrate the presence of hepatitis B surface antigen in these hepatocytes. In our laboratory, we have been unable to demonstrate cytopathic change or increases in antigen titer using standard assay procedures. 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 for up to 14 subpassages and demonstrate the continued presence of specific immunofluorescence with each subpassage. This evidence strongly suggests that hepatitis B virus may indeed be replicating in these liver cells. However, oval cells, as presently constituted, are not appropriate for vaccine development for they represent suspension rather than

monolayer cultures. It would seem of the utmost importance to develop reproducible systems for the development of monolayer cultures of oval cells and then to undertake specific titration assays and neutralization studies neither of which have yet been undertaken.

Methods and Discussion

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 live 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 10 days was established. However, definitive evidence of viral replication was lacking until recent studies documented below. 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.

Recently, we and others have undertaken a new tissue culture system employing human hepatocyte oval cell cultures. Studies in this laboratory and in the laboratory of Drs. M. Watanabe, H. Ohori and N. Ishida have demonstrated the reproducible ability to develop unique oval cell hepatocyte tissue cultures which seem to be capable of maintaining hepatitis B virus growth. Explants of human embryonic liver culture have been the primary source of tissue but recently similar techniques have been applied to young adult human liver tissue and have also yielded hepatocyte growth. Explants were grown in falcon plastic dishes and it was observed that small round cells may be liberated and would float in the surrounding media. These floating cells are immature hepatocytes which have an oval appearance. When these cell cultures are infected with hepatitis B surface antigen rich sera obtained from a patient with fulminant fatal hepatitis, positive immunofluorescent staining is usually demonstrated in the floating oval cells. These cells may be subpassaged for up to 14 passages with persistence of the positive immunofluorescent staining hepatitis B surface antigen. It has been shown by Watanabe, et al, that maximum titers are obtained on day eight after inoculation. In these studies, 12 to 24 week old liver explants were cultured in a growth medium consisting of RPMI 1640 medium with 20% fetal calf

serum and 600 mgm/L bactopeptone, 300 mgm/L glutamine and 160 Mu/L crystalline insulin in a CO_2 incubator at 37°C. The floating cells are collected on the 8th day and centrifuged in Ficol-Coraray for 30 minutes at 1500 rpm. The oval cell rich fraction is removed and may then be infected with hepatitis B surface antigen positive serum. These oval cell cultures in addition to producing albumin have also been shown to produce alpha-1-fetoprotein. Unfortunately, these cultures remain as "floating cultures" or suspension cultures. Successful development of these hepatocytes infected with hepatitis B surface antigen as monolayer cultures have not yet been accomplished. These new techniques, however, give added hope that the cultivation of live hepatitis B virus and hopefully of other hepatitis viruses may have been accomplished albeit not in sufficient form, at this time, for vaccine development.

Conclusions

In the early phases of our work, reproducible and reliable techniques were developed for the short and long term maintenance of hepatocytes in monlayer culture. These hepatocytes were shown to produce albumin and subsequently to produce alpha-1-fetoprotein. These cultures were free of fibroblastic overgrowth and maintained epitheliod characteristics. We were able to sustain persistence of hepatitis B virus for a period of about 10 days but evidence of viral replication using a variety of techniques met with initial failure. Similarly, attempts to grow hepatitis A virus using both standard techniques and "slow" virus techniques" such as co-cultivation, feeder layer cultures and long term maintenance cultures failed to give evidence of viral multiplication. Recently, a new hepatocyte cultivation technique employing the use of oval cells in suspension culture of liver explants has suggested the persistence of hepatitis B antigen when studied by immunofluorescence. These infected oval cells have been subcultured by others for 14 passages suggesting that the eventual cultivation of hepatitis B virus may yet be feasible. Current problems include the conversion of oval cell suspension cultures to a reproducible monolayer system.

Accomplishments to Date

Reproducible and reliable techniques have been developed, both for short and long term maintenance of hepatocytes in monolayer tissue cultures. These cultures are free of fibroblastic cells and retain epithelial characteristics. They can be maintained for periods in excess of 10 weeks and are a likely substrate for the cultivation of hepatitis viruses. The development of this substrate constituted the initial phase of a carefully developed program with the eventual goal being the development of a viral vaccine for hepatitis A and for hepatitis B. The second phase of this program undertook to cultivate hepatitis virus in hepatocyte tissue cultures. This aspect of the program is now actively underway. Initial studies have demonstrated electronmicroscopic evidence of hepatitis B antigen within tissue culture cells during the first week after inoculation. Serologic studies have demonstrated evidence of hepatitis B surface antigen in pour off fluids for periods ranging from 4 to 10 days after inoculation. These encouraging findings led to studies aimed at the long term maintenance of infected oval cell cultures with the eventual goal of demonstrating viral replication in the hepatocytes after a period of intra-cellular viral latency. Hopefully, these studies will provide a second step eventually aimed at the development of both an inactivated and a live attenuated viral vaccine for each of the important forms of hepatitis virus.

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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

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growth 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 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.