Br. J. exp. Path. (1976) **57**, 211.

EVIDENCE FOR THE MULTIPLICATION OF HEPATITIS B VIRUS IN "OVAL CELL" CULTURE ORIGINATED FROM HUMAN EMBRYONIC LIVER

M. WATANABE, T. UMENAI, H. OHORI AND N. ISHIDA

From the Department of Bacteriology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Sendai, 980, Japan

Received for publication 2 October 1975

Summary.—Growth conditions of human oval cells (immature hepatocytes), evidence of hepatitis B (HB) antigen synthesis in oval cells as revealed by immunofluorescent staining and successful passage of such agent in the culture fluid up to the 4th passage are described. The results have been proved to be readily reproducible with different inocula. The oval cells used in these experiments were defined as small round cells, with scant cytoplasm, vesicular nuclei and small nucleoli, vitally stained with indocyanine green and synthesizing α-foetoprotein but not albumin.

MANY attempts have been made to grow the causative agent of hepatitis B (HB) in cell and in organ culture systems, but there is no unequivocal evidence of multiplication *in vitro*. Development of HB antigen in cultured human hepatocytes has been described by Zuckerman, Baines and Almeida (1972) and Noyes (1973). However, either there was no clear evidence of an increase of HB antigen in culture after serial passage or the results have not proved to be readily reproducible.

For the past 3 years our attention has been focused on the establishment of organ culture techniques for hepatocytes originating from human embryonic liver, using Rose's circumfusion chamber (Rose, Kumegawa and Cattoni, 1968). As a result, hepatocytes arranged more or less regularly in clusters reminiscent of the lobular structure of human liver tissue could be maintained for 60 days when examined by the trypan blue dye exclusion test. However, isozyme patterns of LDH shifted from that of the embryonal type to the adult type during the incubation period, suggesting that a differentiation process might have occurred. Almost all of the cells maintained in such cultures showed the typical morphology of hepatocytes and were vitally stained with indocyanine green (Caesar *et al.*, 1961), suggesting that they were outgrowing hepatocytes. When they were infected with a known infective serum containing HB antigen, progressive involvement of hepatocytes was demonstrated by specific immunofluorescent staining in the cytoplasm, but the number of antigen synthesizing cells was limited to about 1 out of every 1000 hepatocytes. These experiments were repeated for about a year, with different inocula and using more than 30 embryos of different ages, but the results were consistent in the low detection rate of antigen synthesizing cells at 1 : 1000. Meanwhile in order to establish an explant type culture of hepatocytes and inspired by the results of Zuckerman *et al.* (1972), the basic conditions necessary to grow hepatocytes were examined. Both mouse embryo and human embryo liver tissues were examined and were found to give rather contradictory results in their nutritional requirements. With human embryo liver, however, when RPMI 1640

medium or McCoy's 5a medium (Noyes, 1973) were used in the presence of 20% foetal calf serum, both inhibited the growth of fibroblasts more than Eagle's MEM medium, resulting in better predominance of hepatocytes. After several preliminary experimental trials, it was found that when RPMI 1640 medium was fortified with bactopeptone, L-glutamine and insulin, the inhibition of fibroblastic growth was excellent. In addition, floating hepatocytes, much smaller in size than mature hepatocytes, appeared in the culture medium. These two cells were distinguished from other cells by vital staining with indocyanine green. When infected, only these small hepatocytes which will be referred to as "oval cells" (Ogawa, Minase and Onoe, 1974) in the text, allowed the synthesis of HB antigen in as high as 20% detection rate within 5 days.

This report concerns the growth conditions of such oval cells, the process of HB antigen synthesis in oval cells as revealed by immunofluorescent staining and the successful passage of the agent in the culture fluid for up to the 3 passages.

MATERIALS AND METHODS

For the selective growth of oval cells growing medium consisting of RPMI 1640 medium (GIBCO) fortified by 600 mg/l of bactopeptone (Difco), 300 mg/l of L-glutamine and 136 μu/ml of crystalline insulin (Sigma), pH 7·0, was used throughout the experiment with added 100 u/ml of penicillin and 100 μg/ml of streptomycin and 20% foetal calf serum. This does not imply that no other medium would allow growth of oval cells or mature hepatocytes in floating condition, but in our limited experience this medium gave the best oval cell growth.

Human livers obtained by abdominal hysterotomy from 12–24 weeks old embryos were used immediately for culture. To date, embryos (34) of 20–24 weeks gave better and more consistent oval cell growth than embryos (15) of 12–16 weeks. The liver tissue was washed with RPMI 1640 medium after the removal of the capsular membrane and cut into fragments approximately 1·0 mm^3 with sharp razors. They were pressed through a wire screen (No. 40) into RPMI 1640 medium using the plunger of a 10-ml plastic syringe. Such tissue fragments were washed 3 times with growing medium not containing serum and sedimented by gravity. Whenever the fragments were collected by light centrifugation, floating oval cells failed to appear in the culture. The settled liver fragments were suspended in growing medium and 1 ml of this suspension was poured into a Falcon plastic dish (diameter 60 mm). When started with a 24 weeks old, 20 g embryo liver, 100 plates could be prepared. To each plate, 4 ml of fresh growing medium was added and the fragments immersed in the medium were incubated at 37° in humidified air supplemented with 5% (v/v) CO_2. After 3 days incubation almost 80% of such fragments were fixed on Falcon dishes and the distance from fragment to fragment was almost equal to the fragment size. Medium exchange was made every 4th day and on the 8th day before the second medium exchange, the fragments were found to be connected with endothelial-like cells adhering to the plate. Surrounded by such endothelial cells, some clusters of hepatocytes could be found. More important, however, is the fact that many floating cells appeared in the medium and 70% of them were identified as immature oval cells (Ogawa *et al.*, 1974) and the remaining 30%, as mature hepatocytes. The former cells did not synthesize albumin but 70% of the latter cells were doing so as judged by immunofluorescent staining. In contrast, 20% of the former cells were synthesizing α-foetoprotein, while almost none of the latter were doing so. Between 8–15 days, oval cells in the floating state revealed a tendency to aggregation by forming clusters; as a result a morphological change occurred: in the cells from spherical to an irregular form, resembling mature hepatocytes. At this stage, they started to synthesize albumin and most of the clusters deposited on the bottom of the culture dish forming cell sheets with a lobular structure.

Infection was performed with known infective HB serum (Jap. No. 3, No. 6 and others) supplied by the Hepatitis Research Committee in Japan. The floating hepatocytes and oval cells in the medium on the 8th day of incubation were withdrawn, centrifuged and sedimented. Floating cells collected from 10 Falcon dishes were combined and mixed with a 2 ml amount of 1 : 5 dilution of infective HB serum in growing medium and each 0·2 ml amount of cell suspensions was returned to 10 Falcon dishes. After one and a half hours of incubation at 37° for virus adsorption, each dish received 4 ml of additional growing medium and was then incubated. This was termed 10^{-2} infection, because the final amount of infective serum was calculated to be 0·04 ml in 4 ml medium.

RESULTS

When direct immunofluorescent staining was carried out with FITC-labelled rabbit antiserum against purified HBs antigen, progressive involvement of the oval cells was demonstrated. Uninfected oval cells were never stained by this procedure and treatment of infected oval cells with rabbit anti-HBs serum prior to addition of the FITC-labelled serum completely inhibited staining of the infected cells, which confirmed that this procedure selectively stained HBs antigen in infected cells. On the 3rd day after infection, antibody attachment was restricted to the perinuclear membrane of oval cells. On the 5th day, however, the cytoplasm of oval cells became filled with homogeneous fluorescent material (Fig. 1). More exactly, when floating cells in culture fluid were removed, collected by centrifugation and examined in smears on glass slides, clusters of fluorescent cells were often encountered and 20% of the oval cell population were judged to be synthesizing HB antigen when Jap. No. 6 (obtained from fulminant hepatitis) was used as the inoculum. On the 7th day, the intensity of the staining and the number of fluorescent oval cells were unaltered. However, evidence of cell destruction ap-, peared. Hepatocytes in clusters attached to the bottom of the dish, were found to be synthesizing HBsAg, but at a limited rate as 1 : 1000.

When such initial infection experiments were repeated, 6 experiments with Jap. No. 3 (fulminant hepatitis), 6 experiments with Jap. No. 6 and 2 experiments with Yoshimitsu serum (from chronic active hepatitis) gave absolutely compatible results. That is, at 10^{-2} infection, Jap. No. 3 gave 10% detection rate, Jap. No. 6, 20%, and Yoshimitsu, 2–3%. When the inoculum No. 6 was diluted in 10-fold, 10^{-1}, 10^{-2} and 10^{-3} dilution it gave 20%, 20% and 5% detection rate, respectively, whereas 10^{-4} up to 10^{-6} dilution gave no response. Thus the sensitivity of the present culture system for HB virus detection seems to be relatively low, although the experiment should be repeated using a well characterized infective serum such as MS-2.

A passage experiment result using the same culture system is illustrated in Fig. 2. When the supernatant fluid of the 5th day culture originated from Jap. No. 6 at 10^{-2} was passaged at 10^{-1} and 10^{-2} dilution, detection rates of 10% and 3% were found by immunofluorescent staining on the 5th day in the second passage. When the supernatant of this second passage material at 10^{-1} obtained on the 5th day was passaged at 10^{-2} dilution, again a 20% detection rate was found in the third passage. Fourth passage was also successful as shown in Fig. 2 and dilution in the end reached 10^{-6} of the original Jap. No. 6. Virtually identical fluorescent changes were found in oval cells in the second, third and fourth passages.

Release of HBs antigen in the culture fluid was examined by the aid of Ausria-II kit (Abbott) and 2–4 fold increase of HBs antigen was detected 3 times in the first infection experiment. The results, however, are of still too preliminary a nature to withstand critical consideration.

DISCUSSION

Various hepatocarcinogens as well as other noxious stimuli including partial hepatectomy have been known to induce proliferation of the so-called " oval cells " in the liver (Ogawa *et al.*, 1974). Proliferation is followed by elevation of α-foetoprotein in the serum, but only for a limited period (Sell, 1974). Moreover, once oval cells have appeared in the liver, they seem to be replaced by mitotic hepatocytes within a few days (Ogawa *et al.*, 1974; Sell, 1974). Although the fate of the proliferated oval cells and the significance of their proliferation are matters of dispute, some investigators consider the possibility of the oval cell being transformed into hepatocytes (Far-

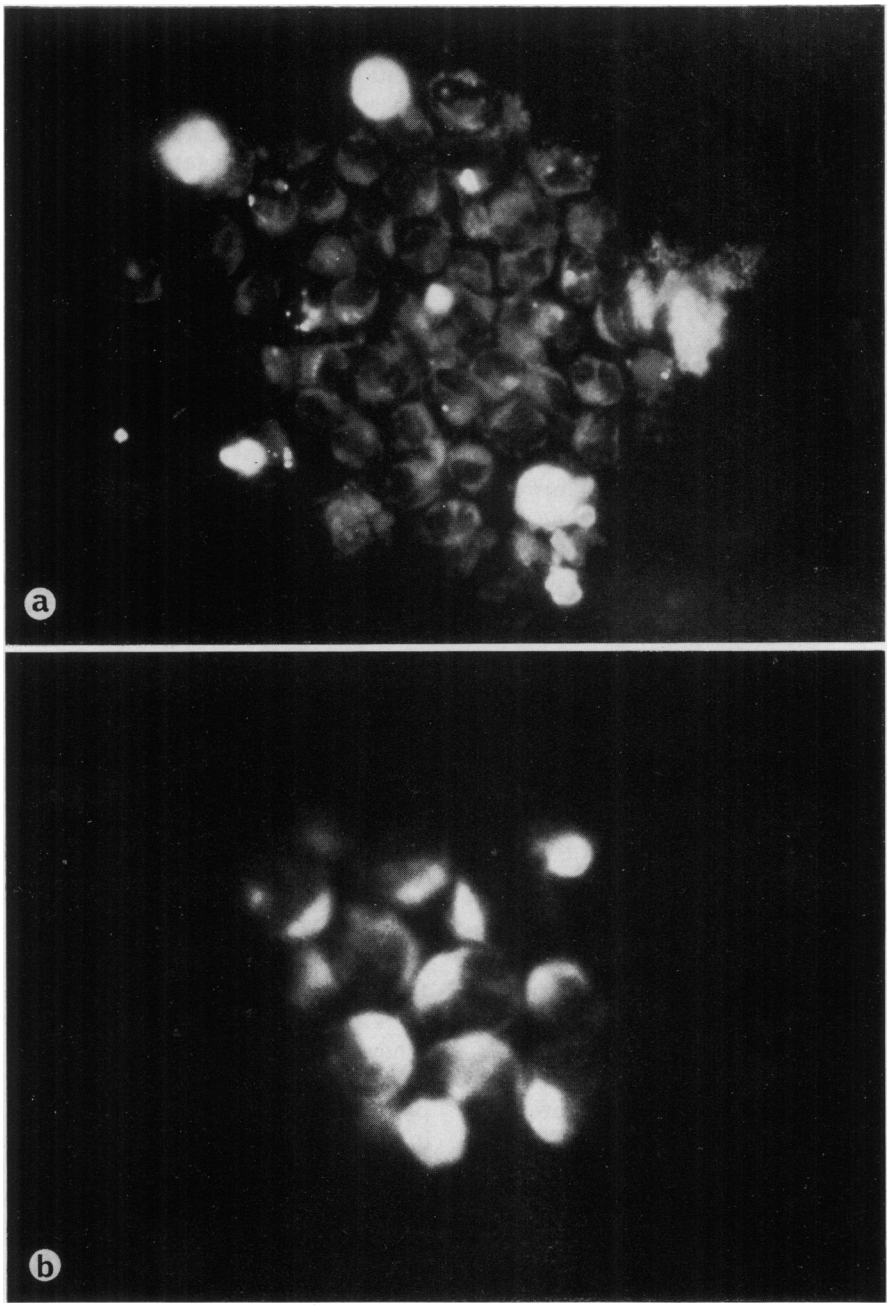

FIG. 1.—Multiplication of HBV in oval cells (immature hepatocytes) originating from human embryonic liver detected by immunofluorescent staining with FITC-labelled rabbit antiserum against purified HBs antigen. (a) Almost 80% of the oval cells in clusters are synthesizing HB antigen. Note the appearance of binuclear cells. (b) On the 5th day after inoculation, figures resembling cap formation are often encountered. ——— Oval cells appeared in the medium 8 days after cultivation of 24 week-old human embryonic liver were infected with HB serum (Jap. No. 6). On the 5th day after inoculation the floating cells in the culture fluid were removed, collected by centrifugation and smears made on glass slides. After fixation with CCl_4, FITC-labelled rabbit antiserum against HBs antigen was added and incubated at 4° overnight. At the end of antigen-antibody reaction, labelled anti-HBs was washed twice with a large volume of cold PBS.

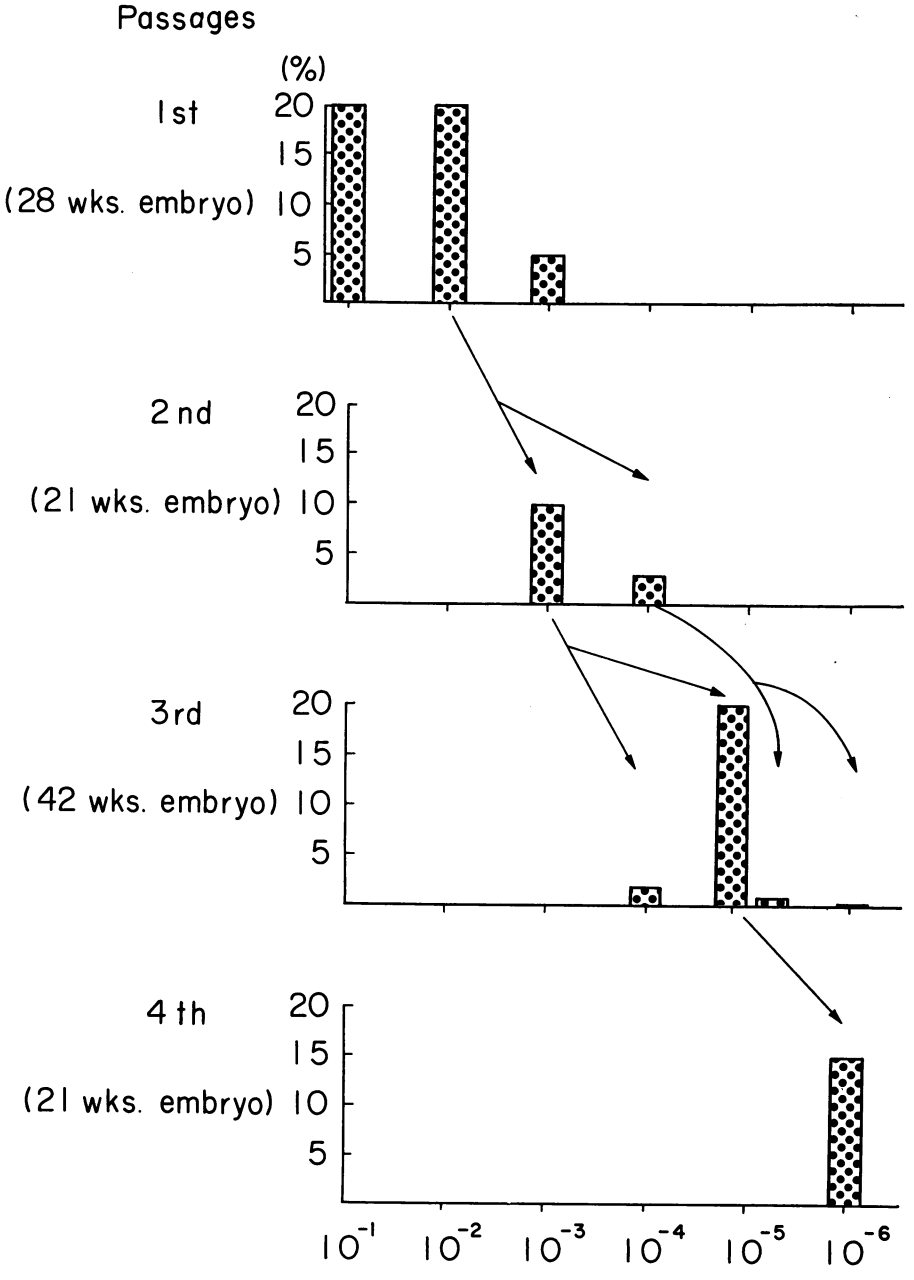

FIG. 2.—A passage experiment through the same culture system described in Fig. 1.
Ordinate: fluorescent cells percent.
Abscissa: final dilution of the inoculum (Jap. No. 6).

ber, 1956), although others did not agree (Rubin *et al.*, 1963). The oval cells in this culture were defined as small round cells with scant cytoplasm, vesicular nuclei and small nucleoli. They are not in complete agreement with the descriptions of oval cells found *in vivo* (Ogawa *et al.*, 1974; Farber, 1956). However, indocyanine green incorporation (Caesar *et al.*, 1961) in such cells was taken as evidence of them being classed as hepatocytes, probably immature, based upon our experience of vital screening of various cells (unpublished). Although the histopathological studies so far conducted with biopsy and autopsy liver specimens of HB patients do not support a specific participation of such oval cells in the growth of HB virus, our finding of a low detection rate (1 : 1000) of antigen synthesizing cells among adult type hepatocytes in Rose's circumfusion chamber may support the hypothesis that HB virus can grow best in such undifferentiated hepatocytes.

The aetiological agents of molluscum contagiosum and skin warts, hepatitis A and B virus in liver and adult epidemic gastroenteritis in the intestine have not been successfully grown even using human diploid cells *in vitro*. The fact that these agents are proliferating *in vivo* in the tissues where differentiation is occurring should be a matter of future concern.

This work was supported partly by a Grant in Aid for Scientific Research from the Ministries of Education and Welfare, Japan.

REFERENCES

CAESAR, J., SHALDON, S., CHIANDUSSI, L., GUEVARA, L. & SHELOCK, S. (1961) The Use of Indocyanine Green in the Measurement of Hepatic Blood Flow and as a Test of Hepatic Function. *Clin. Sci.*, **21**, 43.

FARBER, E. (1956) Similarities in the Sequence of Early Histological Changes Induced in the Liver of the Rat by Ethionine, 2-Acetylaminofluorene, and 3′-Methyl-4-dimethylaminoazobenzene. *Cancer Res.*, **16**, 142.

NOYES, W. F. (1973) Hepatitis B Virus Antigen Development in Cultured Human Hepatocytes. *Proc. Soc. exp. Biol. Med.*, **144**, 845.

OGAWA, K., MINASE, T. & ONOE, T. (1974) Demonstration of Glucose 6-phosphatase Activity in the Oval Cells of Rat Liver and the Significance of the Oval Cells in Azo Dye Carcinogenesis. *Cancer Res.*, **34**, 3379.

ROSE, G. G., KUMEGAWA, M. & CATTONI, M. (1968) The Circumfusion System for Multipurpose Culture Chambers. *J. Cell Biol.*, **39**, 430.

RUBIN, E., HUTTERER, F., DANON, G. & POPPER, H. (1963) Deoxyribonucleic Acid Turnover in a Heterogeneous Population. Life Span of Hepatic Ductular Cells. *Lab. Invest.*, **12**, 657.

SELL, S., NICHOLS, M., BECKER, F. F. & LEFFERT, J. L. (1974) Hepatocyte Proliferation and α_1-Fetoprotein in Pregnant, Neonatal and Partially Hepatectomized Rats. *Cancer Res.*, **34**, 865.

ZUCKERMAN, A. J., BAINES, P. M. & ALMEIDA, J. D. (1972) Australia Antigen as a Marker of Propagation of the Serum Hepatitis Virus in Liver Cultures. *Nature, Lond.*, **236**, 78.