Hepatitis B Virus Antigen Development in Cultured Human Hepatocytes[1] (37695)

WILBUR FISKE NOYES

Roswell Park Memorial Institute, Buffalo, New York 14203

Australia antigen, discovered by Blumberg *et al.* in 1965 (1) and now generally referred to as hepatitis B antigen or HB antigen, has been shown to be closely associated with the presence of the infective agent (2–4), and has been used as an indicator of the presence of the serum hepatitis virus (hepatitis B virus). There have been a number of recent attempts to propagate the hepatitis B virus in cell or organ culture systems using HB antigen as an indicator of the infectious agent (5–10). However, either there was no clear evidence of an increase of HB antigen in culture over that originally added or the results have not proven to be readily reproducible (7, 8). Thus, it does not appear that production of HB antigen *in vitro* has been conclusively demonstrated.

The present communication describes the development of HB antigen in cultured human fetal hepatic parenchymal cells as demonstrated by immunofluorescence methods.

Materials and Methods. Tissue. Human fetal liver was obtained by abdominal hysterotomy. Fetal ages varied from about 12 to 18 weeks and crown to rump measurement from 7 to 14 cm. The liver was removed and placed immediately in McCoy's 5a (Microbiological Associates, Bethesda, MD) containing 10% fetal bovine serum at 4° for transportation to the laboratory. Elapsed time from delivery of the fetus to receipt of the fetal liver was 1–2 hr.

Cultures. The fetal liver was pressed through a fine stainless steel wire screen into Hanks' balanced salt solution (BSS) using the plunger of a 5-ml plastic syringe. The wire screen was No. 40 and had an open area of 0.4 mm^2. The tissue fragments produced by this means had a diameter of 0.3–0.5 mm and were washed twice in BSS by gravity settling for 2 min in a 15-ml centrifuge tube. The settled suspension of liver fragments (0.1–0.2 ml) was added to T-30 flasks (Bellco, Vineland, NJ) in 2 ml of media consisting of McCoy's 5a plus 10% fetal bovine serum. The T-flasks contained 4 coverslips (11 × 35 mm) coated with bovine collagen (11). After addition of the liver fragments to the T-flasks, they were gassed with 5% CO_2 in air and incubated overnight at 37° in a horizontal position to allow attachment of the liver fragments. Eighteen hours after initiating the cultures, the volume of media was brought to 4 ml and the flasks placed on a rocking apparatus as described by McLimans (12). The flasks were rocked through 50° every 3 min, thus maintaining a thin film of media over the cultured tissue. Alternatively, with comparable results, the flasks were placed on a roller apparatus (Wheaton Vitroller, Millville, NJ) and rotated at 14 revolutions per hour. The culture media was changed as dictated by pH, generally twice per week.

Acute-phase sera. Acute-phase sera containing HB antigen from patients hospitalized with hepatitis and antigen-negative sera from blood donors were used in these studies. The sera were checked for the presence of HB antigen by counterelectrophoresis (13) and agar gel diffusion (14), using guinea pig antisera against HB antigen and antigen-positive and antigen-negative control sera (Abbott Laboratories, N. Chicago, IL).

Antisera. Guinea pig antisera against hepa-

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titis B antigen (Abbott Labs.) was used as a basis for localization of the antigen. The guinea pig antisera was prepared by injections of concentrated and purified HB antigen from selected human plasma and gave lines of identity to Australia-1 antiserum (15). The fluorescein-labeled and unlabeled guinea pig antisera against HB antigen also gave lines of identity to HB antigen reference antisera from the Research Resources Branch, National Institute of Allergy and Infectious Disease in tests conducted in this laboratory. Three reagents were used for immunofluorescent localization of the antigen: (i) fluorescein isothiocyanate-labeled guinea pig antisera against HB antigen, supplied through the courtesy of Abbott Laboratories; (ii) guinea pig antisera against HB antigen coupled with fluorescein isothiocyanate by the method of Marshall *et al.* (16) and purified by gel filtration on Sephadex G-25; and (iii) unlabeled guinea pig antisera against HB antigen used in the indirect staining method and poststained with fluorescein-labeled rabbit anti-guinea pig globulin (Hyland, Costa Mesa, CA). The three methods gave comparable results, although the direct method was preferred, due to the low level of nonspecific fluorescence.

Staining procedure. Coverslip cultures were washed in pH 7-buffered saline, dried in air, and fixed for 10 min in acetone. Frozen sections of liver were air dried and fixed in acetone for 10 min. For the direct immunofluorescence method, coverslip cultures or sections were stained for 30 min at room temperature with labeled antisera, washed twice with buffered saline, and mounted in pH 7-buffered glycerol. For the indirect method, sections or coverslips were stained with unlabeled guinea pig antisera against HB antigen for 30 min followed by 2 washes with buffered saline, then stained with fluorescein-labeled rabbit anti-guinea pig globulin (absorbed with mouse liver powder and diluted 1:8) for 30 min, washed twice, and mounted. The collagen coating of the coverslips did not interfere with fluorescence microscopy aside from an occasional blue fluorescence.

Specificity tests. Tests for the specificity of the fluorescent antibody staining included absorption and blocking experiments. Prestaining with unlabeled guinea pig antisera to HB antigen significantly reduced the level of fluorescence when subsequently stained with labeled antisera. Prestaining with normal serum did not have this effect. Absorption of the fluorescein-labeled antisera with lyophilized human serum containing HB antigen abolished fluorescence. Absorption with lyophilized antigen-negative human serum did not have an effect on the level of fluorescence. Controls on the specificity of the indirect procedure included absorption of the unlabeled antiserum with specific-antigen-containing serum, prestaining with normal sera, and poststaining with labeled unrelated antisera. Examination of frozen sections of liver from a kidney transplant patient with long-term HB antigenemia by direct and indirect immunofluorescence methods revealed characteristic cytoplasmic and nuclear localization of HB antigen, similar to that described by Nowoslawski *et al.* (17) and Madalinski *et al.* (18). Sections of the liver of persons negative for HB antigen were negative.

Exposure of cultures to acute-phase sera. The fragments were exposed to HB antigen-positive or antigen-negative sera diluted 1:10 in BSS for 1 hr at 37° in Wasserman tubes, with shaking every 5 min. All fluid was then removed and the liver fragments placed in T-flasks in 2 ml of media consisting of McCoy's 5a plus 10% fetal bovine serum. The flasks were incubated overnight in the horizontal position to allow attachment of the fragments.

Results and Discussion. After overnight incubation in the horizontal position, the majority of fetal liver fragments attached to the collagen substrate, and at 3–5 days, an outgrowth of cells was visible surrounding the fragments. Microscopically, the cellular outgrowth was made up of sheets and cords of cells with a regularly polygonal structure and a very granular cytoplasm in the unstained state and containing a spherical nucleus with 1 or 2 spherical nucleoli. The outgrowing cells displayed the typical morphology and arrangement of differentiated hepatic parenchymal cells (hepatocytes) as observed in sections of human fetal liver fixed on receipt. Glycogen was readily demonstrat-

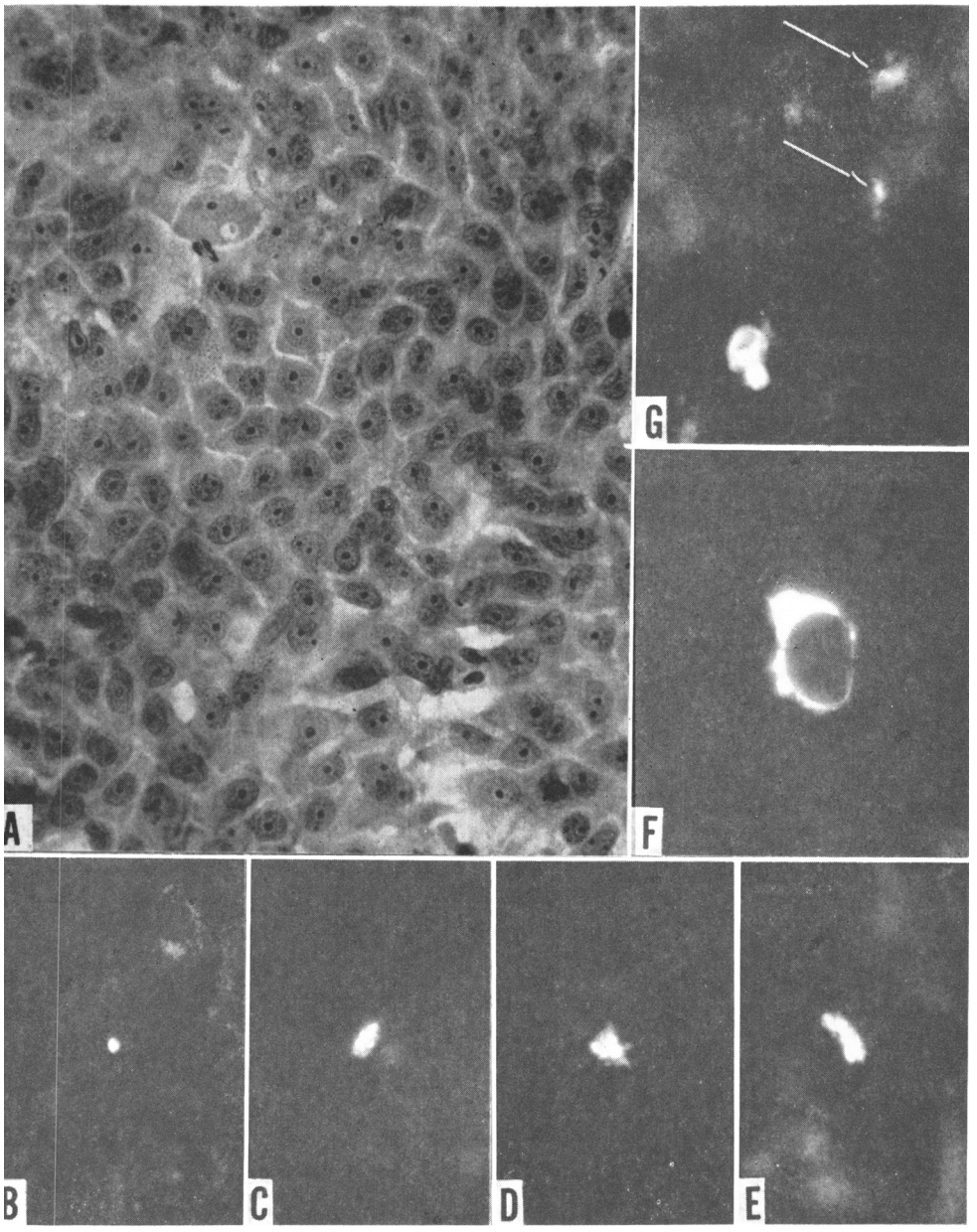

FIG. 1A. Hepatocyte outgrowth from human fetal liver after 21 days culture. H and E, 250×.

B. Hepatitis B antigen localized in nucleus of hepatocyte in outgrowth zone of colony 37 days after exposure to acute-phase serum. Immunofluorescence, 250×.

C. Granular hepatitis B antigen in nucleus of hepatocyte in outgrowth zone 28 days after exposure to acute-phase serum. Immunofluorescence, 1000×.

D. Hepatitis B antigen in hepatocyte nucleus in outgrowth zone 14 days after exposure. Immunofluorescence, 400×.

E. Hepatitis B antigen in hepatocyte nucleus 34 days after exposure. The nuclear boundary of the cell can be discerned. Immunofluorescence, 630×.

F. Intensely-staining hepatitis B antigen filling part of cytoplasm of hepatocyte in outgrowth zone of colony 38 days after exposure. Immunofluorescence, 630×.

G. Hepatocytes containing HB antigen in explant area of culture. Shrunken cell at lower left contains cytoplasmic antigen and two cells at upper right (arrows) contain nuclear antigen, 28 days after exposure. This is suggestive of cell to cell transfer of antigen. Immunofluorescence, 400×.

ed in the cells as described previously (11). The area of hepatocyte outgrowth continued to increase for 10–14 days, and mitotic figures were readily observed in the cells during this period. Figure 1A illustrates the appearance of the outgrowth of hepatocytes from a liver explant after 21 days of culture. Note the polygonal structure of the cells and the spherical nuclei containing 1 or 2 nucleoli. Areas of formation of cells in the granulocytic and erythrocytic series were prominent in the first 2 weeks of culture and were largely confined to the explanted tissue. Fibroblastic growth was controlled in the cultures by the addition of 20 μg/ml of hydrocortisone (Merck, West Point, PA) to the media. The fetal hepatic parenchymal cells could be maintained in culture in good morphological condition for periods of at least 60 days (11).

Cultures were examined for the presence of HB antigen the day following exposure to antigen-positive or antigen-negative sera and subsequently at twice-weekly intervals. One-third of a coverslip (approximately 1 cm^2) was removed for these examinations. Four different hepatitis B antigen-positive acute-phase sera from patients with hepatitis were found capable of inducing the development of HB antigen in the cultured fetal human hepatocytes. None of the control sera not containing antigen induced development of antigen in the hepatocytes. The antigen was observed only in hepatocytes, either in the original liver explant or in the outgrowth area of the colony. Antigen was not observed in fibroblasts surrounding the hepatocyte colonies.

The immunofluorescence-localized HB antigen was first detected 14 days after exposure and was limited initially to the nucleus of the hepatocyte. The antigen occurred as irregularly shaped deposits of varying size, for the most part of a granular nature. Figures 1B–E illustrate the variety in size and shape of nuclear-localized antigen. The variation in size of the antigenic area is obvious when the magnification of the figures is considered (Fig. B 250×, C 1000×, D 400×, E 630×). In almost all cases, the antigen occurred in hepatocytes with clear cytoplasm and low autofluorescence, so that the nuclear boundaries are not obvious in most of the figures. The cell illustrated in Fig. 1E is an exception, and the nuclear boundaries can be clearly discerned. Nuclear hepatitis B antigen developed in cultured hepatocytes from fetuses of 12-, 14-, and 18-weeks gestation. Fourteen days after exposure to acute-phase sera, nuclear antigen was detected in 1 or 2 cells in the approximately 1-cm^2 culture area examined. Twenty days to four weeks postexposure, 1–6 hepatocytes with nuclear-localized HB antigen were detected, and 5–6 weeks following exposure, nuclear antigen was observed in 2–17 widely separated hepatocytes in the culture area examined. Beyond 6 weeks, a decreased number of hepatocytes with nuclear antigen was noted, and at 9 weeks, none were detected. Table I summarizes the number of cultured hepatocytes demonstrating nuclear HB antigen according to weeks after exposure. The nuclear HB antigen observed in some of the cultured hepatocytes in the present study is similar to the immunofluorescence-localized nuclear HB antigen demonstrated by Nowoslawski *et al.* (particularly Figs. 1 and 2A) in sections of liver from patients with lymphoproliferative disorders and HB antigenemia (17), and appears to be similar to the nuclear HB antigen described by Coyne, Blumberg, and Millman (8) in cells from liver biopsies of patients with HB antigenemia, detected with fluorescein-labeled antibody.

The observations that HB antigen developed initially in the nuclei of cultured hepa-

TABLE I. Number of Cultured Hepatocytes Demonstrating Nuclear or Cytoplasmic Localization of HB Antigen in 1-cm^2 Culture Area.

Weeks postexposure	Nuclear	Cytoplasmic[a]
1	0	0
2	1,2	0
3	5,4	0
4	1,6,1,2	1,1
5	5,2,10,10,17	1,4,3,1
6	2,2,5,4,4	4,2,1
8	1,1,2	2,8
9	0	1,7

[a] Multiple antigen-containing cells occurred as foci.

tocytes provides support for the concept that the hepatocyte nucleus is the primary site of viral replication. Huang (19) has previously suggested that the nucleus of the hepatocyte is the primary site of multiplication of hepatitis B virus, from electron microscopic observations of characteristic approximately 20-nm viral-like particles in hepatocyte nuclei of specimens from patients with HB antigen-positive hepatitis. Nowoslawski *et al.* (17) and Nelson, Barker, and Danovitch (20) have also observed the characteristic viral-like particles in liver cell nuclei of patients with antigen-positive lymphoproliferative disorders or acute antigen-positive hepatitis. It has recently been determined, using immuno-electron microscopy, that the 20-nm nuclear viral-like particles have the specificity of HB antigen (21).

Hepatitis B antigen was first observed in the cytoplasm of the cultured hepatocytes 28 days following exposure, and at this time was limited to single cells in the culture area examined. Five to six weeks after exposure, cytoplasmic-antigen-containing cells were detected in increased numbers, occurring singly and as foci of 2–4 cells. These foci of antigen-containing cells increased slowly in size and at 8 and 9 weeks consisted of clusters of 2–8 cells; single cells with cytoplasmic antigen could also be found at this time. Observations were not made beyond 9 weeks. Table I summarizes the number of cultured hepatocytes demonstrating cytoplasmic HB antigen according to weeks following exposure. Cytoplasmic antigen was frequently observed to be of a lumpy or granular nature, but also occurred as diffusely staining antigen. The development of cytoplasmic antigen was observed only, with one exception, in cultured hepatocytes from an 18-week fetus and not in hepatocytes from 12- and 14-week fetuses. The one exception was the observation of HB antigen in the cytoplasm of 1 cultured hepatocyte from a 12-week fetus. An example of cytoplasmic HB antigen is illustrated by Fig. 1F, in which the cytoplasm of the hepatocyte is partially filled with diffuse, intensely staining antigen 38 days after exposure to acute-phase serum. Cytoplasmic hepatitis B antigen in hepatocytes of patients with HB antigenemia, demonstrated by immunofluorescence methods, has been observed by a number of workers (17, 22, 23).

The present observations of the initial development of HB antigen in the nucleus of the cultured fetal hepatocyte and subsequent development of antigen in the cytoplasm may imply a secondary cytoplasmic stage in the formation of the hepatitis B virus, and may support to some extent the view held by several workers that the nuclear 20-nm particle gains a coat in the cytoplasm, forming the 42-nm Dane particle, theorized to be the complete infectious virus (24–26). It is also possible that the nuclear antigen observed in this study represents the infectious virus and that the later-developing cytoplasmic antigen is nonstructural. This would be consistent with the findings that the 20-nm viral-like particles are found predominantly in the hepatocyte nucleus and that Dane or other particles have not been demonstrated in significant numbers in hepatocytes (17–21). Furthermore, the filtration studies carried out by McCollum using human volunteers (27) indicate a size of less than 26 nm for the infectious agent. Electron microscopic studies of cultured antigen-producing hepatocytes should help to clarify this issue.

Cell to cell transfer of HB antigen may occur in culture. This is suggested by the development of foci or plaques of hepatocytes with cytoplasmic antigen, as described above. Nowoslawski *et al.* (17) have described the focal nature of HB antigen-containing hepatocytes in sections of liver from patients with lymphoproliferative disorders and HB antigenemia. The focal distribution of HB antigen-containing parenchymal cells in frozen sections of liver from a kidney transplant patient with long-term HB antigenemia has also been observed in this laboratory, using fluorescein-labeled antibody. Cell to cell transfer of HB antigen is also suggested by observations of arrangements of antigen-containing cells as illustrated by Fig. 1G. This figure illustrates a group of hepatocytes observed 28 days postexposure. The shrunken cell at lower left contains cytoplasmic antigen, and two cells at the upper right (arrows) contain nuclear antigen. Since the development of nuclear antigen occurs at an

earlier stage, this is suggestive of a transfer of antigen from the cell with cytoplasmic antigen to the adjacent cells displaying nuclear antigen. The antigen may possibly spread through intracellular contacts, similar to that demonstrated for vaccinia virus in cultures of human epidermoid carcinoma using fluorescein-labeled antibody (28).

The maximum number of cultured hepatocytes developing HB antigen was approximately 1 in 10^3–10^4 cells. Calculation of the number of infective particles in the human acute-phase sera to which the cultures were exposed, from the maximum number of hepatocytes developing HB antigen, gives approximately 6×10^3 infective particles per ml. This is low by a factor of about 100 from the number of infective particles of hepatitis B virus in serum determined by Murray (29) in human volunteers. This lack of correlation may be due to a blocking effect from the large number of noninfective particles present in the acute-phase sera to which the cultures were exposed. Murray estimated the concentration of infective particles in acute-phase sera to be 10^6/ml by titration in human volunteers (29), and the concentration of HB antigen in similar acute-phase sera as determined by electron microscopy is usually greater than 10^{10}/ml (30). Thus, the ratio of noninfective to infective particles in the culture inoculum was probably approximately 10^4:1. The small number of hepatocytes producing HB antigen in culture could also be due to a limited capacity of hepatocytes from fetuses in an early stage of development to support the synthesis of HB antigen. This is consistent with the observations that the secondary development of HB antigen in the cytoplasm of the hepatocytes was almost exclusively observed in cultured hepatocytes from the oldest fetus. Fetuses infected *in utero* at 7–8 months are capable of supporting replication of hepatitis B virus, as demonstrated by the presence of HB antigen in cord serum and in postnatal serum from infants of mothers who had acute type B viral hepatitis during pregnancy (31). Thus, hepatocyte cultures from fetuses 6 months or older may support production of HB antigen to a greater extent than that described in this study.

Summary. Australia antigen, now termed hepatitis B antigen or HB antigen, has been demonstrated to be closely associated with the serum hepatitis virus and to be an indicator for the presence of the infective agent. The development of HB antigen in cultured human fetal hepatic parenchymal cells (hepatocytes) after exposure to acute-phase sera containing the antigen is described. Hepatitis B antigen was demonstrated in the cultured cells by immunofluorescence methods and specificity determined by agar gel diffusion, counterelectrophoresis, blocking and absorption experiments, and examination of frozen sections of liver containing the antigen. Development of HB antigen in the cultured fetal hepatocytes was slow and occurred in a limited number of cells. Hepatitis B antigen was detected initially as irregularly shaped granular deposits in the nuclei of the hepatocytes and was first observed 2 weeks after exposure. These observations support the concept of the hepatocyte nucleus as the primary site of viral replication. Hepatitis B antigen was observed in the cytoplasm of cultured hepatocytes beginning 4 weeks following exposure. Five to six weeks after exposure, cytoplasmic-antigen-containing hepatocytes were detected in increased numbers, occurring as foci or plaques of 2–4 cells. The foci of antigen-containing cells continued to increase slowly in size. These findings may imply a secondary cytoplasmic phase in the development of hepatitis B virus and also suggest cell to cell transfer. The small number of cultured hepatocytes in which HB antigen developed may be due to a limited capacity of hepatocytes from fetuses in early stages of gestation to support viral synthesis, or to a blocking effect exerted by the large ratio of noninfective to infective virus particles present in the inoculum.

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