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Effect of Hepatitis A Virus Infection on Cell Metabolism *in Vitro* (41757)

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Abstract. Hepatitis A virus (HAV), when inoculated into cultures of the PLC/PRF/5 cell line which produces the surface antigen of hepatitis B virus (HBsAg), showed growth characteristics different from those of other picornaviruses. Antigen of HAV (HAAg) is expressed only about 10 days after infection. No major impact on the overall macromolecular biosynthesis of the host cells is observed. The growth rate of HAV-infected and uninfected cells was comparable, although the plating efficiency of infected cells was lower. Different hormonal factors were tested for their ability to stimulate viral antigen expression. Dexamethasone or prostaglandin E_1 added to the culture medium increased HAAg expression; insulin reduced expression. Persistent infection of hepatoma cells by HAV never led to a cytolytic infection. In temperature-shift experiments, an adverse effect on the expression of HAAg and HBsAg was observed. In all experiments, the amounts of HBsAg in HAV-infected cells were reduced. On the whole, no major influence on host-cell metabolism is observed in cells persistently infected with HAV. Cell-mediated immunological response as a mechanism of pathological changes in HAV-infected liver is, therefore, more likely than a cytopathological effect.

Propagation of hepatitis A virus (HAV) in tissue culture has been described for a variety of cell types, among which were kidney and hepatoma cells and fibroblasts (1–5). These culture systems offer both the possibility of producing large amounts of viral antigen for diagnostic tests, and the opportunity of studying host–virus interactions *in vitro*. From its biophysical characteristics, HAV has been classified as an enterovirus (type 72) but its rate of replication *in vitro* is quite different from other picornaviruses (6). The time before viral antigen (HAAg) appears is very much longer than that observed for most other picornaviruses and HAV causes no obvious cytopathological changes in infected cells. In extracellular spaces, HAAg could be detected only in minute amounts. The goals of this study were to investigate in detail the atypical interaction between host cells in culture and HAV, and to stimulate viral expression by changes in cell metabolism.

Materials and Methods. *Virus.* The inoculum used for infection (from patient MBB) was adapted to cell culture through eight passages (2).

Cell culture. The cell line PLC/PRF/5, derived from a primary hepatocellular carcinoma, synthesizes α-fetoprotein (AFP) and hepatitis B virus surface antigen (HBsAg) among other liver specific proteins (7). The cell line has been subcloned (8). Uninfected cells were cultivated in 25-cm^2 flasks (Nunc) at 37°C in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS). For virus inoculation, 1 ml of freeze-thawed cell extract from infected cells (10^5 $TCID_{50}$) was incubated with the confluent cell layer for 4 hr at 37°C. For maintenance, infected cells were incubated in MEM supplemented with 2.5% FCS. As serum-free medium, Iscove's modification of Dulbecco's medium (Flow Laboratories) was used. Insulin (CR) (Hoechst), prostaglandin E_1 (Sigma) and dexamethasone (Fortecortin) (Merck) were added to the medium in different experiments. HAAg, HBsAg, and AFP were extracted from cells by repeated freezing and thawing, and appropriate aliquots were tested by radioimmunoassay (RIA). For determination of HBsAg, the AUSRIA test of Abbott, Chicago was used. HAAg was tested in a solid-phase radioimmunoassay. Briefly, 100 μl of cell extracts were incubated overnight with polystyrene beads which were coated with human anti-HAV. After washing, a second incubation with ^{125}I-labeled anti-HAV followed. Radioactivity was measured in a γ-counter. Protein concentration was measured by Lowry's method using bovine serum albumin as standard. Cell number was determined with a hemocytometer.

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Pulse labeling of cells. HAV-infected and mock-infected cells were incubated with medium containing [^{3}H]uridine or [^{3}H]thymidine (20 μCi/ml, Amersham) for 4 hr. Media were supplemented with 2.5% dialysed FCS. After incubation with radioactive precursors, cells were washed thoroughly and disrupted by repeated freezing and thawing. Overall RNA and DNA synthesis was determined by precipitation of labeled macromolecules with 10% TCA. Precipitates were collected on filter paper (Whatman 3MM) and washed with 10% TCA. Radioactivity was determined in a liquid scintillation counter.

Results. *I. Influence of HAV on cell growth and biosynthesis of macromolecules.* Chronically infected, HAAg-producing and mock-infected PLC/PRF/5 cells were suspended by trypsinisation and plated at a density of 3.0 $\times$ 10^4 cells/cm^2. A growth curve was made by determination of cell counts and total protein content in triplicate culture flasks. HBsAg and HAAg were extracted from the cells and quantified by RIA. The plating efficiency of infected cells was considerably lower (63%) than that of uninfected cells (80%). This was seen both by measuring total cell protein (Fig. 1a) as well as by determining cell number (data not shown). Following attachment of the cells, the rate of growth was similar in infected and uninfected cells, suggesting that the capacity of viable cells to divide has not been altered by the virus. Figure 1b shows the relative amounts of HBsAg and HAAg per microgram total cell protein. During the first 3 days after plating, the relative amounts of HAAg were increasing while those of HBsAg decreased. After day 3, HAAg leveled off and HBsAg rose slightly. In mock-infected cells the amounts of HBsAg extracted from the cells reached saturation levels 5 days after plating.

In order to investigate the effect of HAV on the biosynthesis of cellular macromolecules in a confluent monolayer, HAV-infected and uninfected PLC/PRF/5 cells were pulse-labeled with RNA or DNA isotope-labeled precursors. Incorporation into macromolecules was determined by measuring TCA precipitable counts (Table I). Neither in the early phase after infection when no viral antigen is yet expressed, nor in the later phase when HAAg can be detected, was a significant difference detected in the absolute and relative amount of TCA-precipitable counts between infected and uninfected cells, indicating that the virus has no effect on gross RNA or DNA synthesis of infected PLC/PRF/5 cells.

II. Effect of modulation of cell metabolism on viral antigen expression. From the results mentioned above, it was concluded that in-

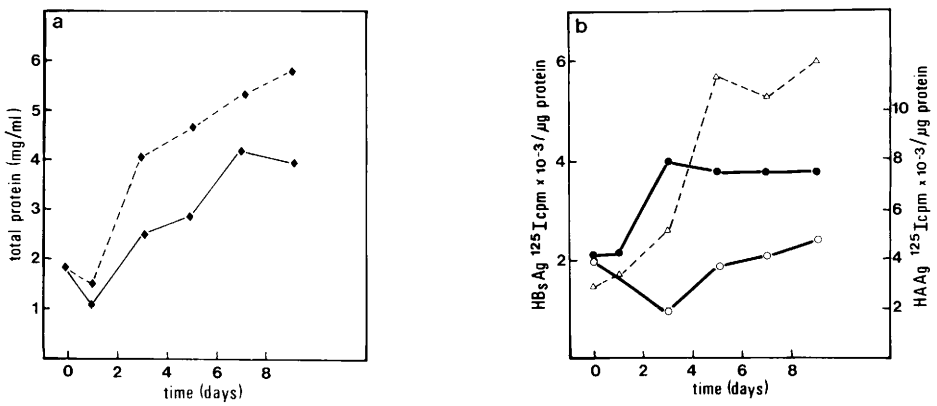

FIG. 1. Growth curve of HAV-infected and uninfected PLC/PRF/5 hepatoma cells. Infected, HAAg-producing and uninfected cells were trypsinized 4 weeks after virus inoculation and seeded at the same cell density in 25-cm^2 flasks. At the time indicated after cell passage, three flasks were trypsinized, the cells were suspended in 1 ml PBS and cell counts, protein content, HAAg, and HBsAg were determined. The data show one of three experiments. (a) Total cell protein in mg/ml; (- - -) PLC/PRF/5 control cells; (——) PLC/PRF/5 cells infected with HAV. (b) HAAg and HBsAg determined by RIA, expressed in cpm/μg protein; (△) HBsAg of uninfected cells; (○) HBsAg of HAV-infected cells; (●) HAAg of HAV-infected cells.

TABLE I. INCORPORATION OF [^{3}H]THYMIDINE AND [^{3}H]URIDINE INTO NUCLEIC ACIDS OF HAV-INFECTED AND NONINFECTED PLC/PRF/5 CELLS

Time after infection (days)	[^{3}H]Thymidine incorporation (cpm/mg protein)		[^{3}H]Uridine incorporation (cpm/mg protein)	
	HAV-infected	Mock-infected	HAV-infected	Mock-infected
1	580	523	153	102
7	723	982	65	41
15	125	89	147	62
30	311	n.d.	32	111

Note. HAV-infected and mock-infected cells were pulsed for 4 hr at the indicated time after infection. TCA-precipitable counts and protein content were determined. Data are expressed as cpm/mg protein. On average, a variation between duplicate cultures of 8% was observed.

fection by HAV does not exert a major influence on overall host-cell metabolism, as a consequence of which the virus can persist in the culture for long periods of time. As described before (2, 4), HAV replicates in cell culture at a rather slow rate, so that viral antigen expression can be detected only about 10–14 days after infection with a highly infectious inoculum (10^5 $TCID_{50}$). The following experiments were designed to influence the balance between virus replication and host metabolism by altering certain parameters in host-cell maintenance. Various growth conditions as well as a variety of hormones were used.

1. Influence of temperature on virus replication. PLC/PRF/5 cells were infected with HAV of the 12th passage in cell culture at a multiplicity of about 1. Half of the cells were cultivated at 37°C, the other half at 32°C. Twenty-four days after infection, the temperature was shifted from 37 to 32°C and vice-versa. Figure 2a depicts the amount of HAAg and Fig. 2b the amount of HBsAg extracted from the cells over the course of the experiment. AFP was not detected. HAAg was found already 10 days after infection when cells were cultured at 37°C. After about 30 days, antigen production reached a saturation level. At 32°C culture temperature, HAAg was hardly detectable. However, when conditions were shifted to the more favorable temperature (24 days after infection), HAAg production was markedly stimulated, indicating that the virus had survived in a cryptic state at 32°C but could be activated to express its antigen at 37°C. HBsAg expression is favored at 32°C (Fig. 2b) in HAV-infected as well as in uninfected cells (not shown).

2. Hormonal factors. Expression of hepatic cell function by hepatocytes *in vitro* is influenced by the presence of hormones and growth factors. We were, therefore, greatly interested whether the expression of HAAg by infected PLC/PRF/5 cells can be stimulated by factors that favor the metabolism of hepatocytes. First, we tested the importance of serum concentration in culture medium, and then individual components (insulin, glucocorticoid,

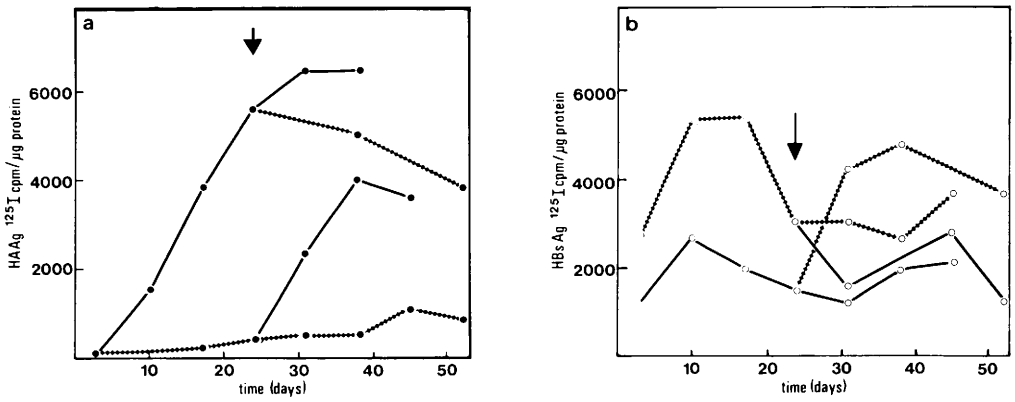

FIG. 2. Temperature dependence of expression of (a) HAAg and (b) HBsAg in HAV-infected PLC/PRF/5 cells. HAV-infected cells were cultivated at 32 and 37°C, respectively. Twenty-four days after infection a temperature shift was performed (↓). Aliquots of the cell lysates were tested for HAAg and HBsAg by RIAs. (——) 37°C; (- - -) 32°C.

TABLE II. EFFECT OF INSULIN AND FCS ON ANTIGEN EXPRESSION IN HAV-INFECTED AND MOCK-INFECTED PLC/PRF/5 CELLS

Supplements to Iscove's modification of Dulbecco's medium	HAV-infected cells		Uninfected cells	Total cell protein (mg/ml)	
	HAAg (cpm/μg protein)	HBsAg (cpm/μg protein)	HBsAg (cpm/μg protein)	HAV-infected	Uninfected
None	3465	3514	3572	2.88	2.72
2.5% FCS	9322	4696	5323	1.44	1.40
10% FCS	4030	4596	4623	1.92	1.92
Insulin (3×10^{-5} *M*)	1617	3015	2900	4.00	4.08

and prostaglandin) for their effect on HAAg and HBsAg expression in HAV-infected PLC/PRF/5 hepatoma cells. In preliminary experiments, we showed that Iscove's modification of Dulbecco's medium is sufficient to maintain quiescent HAV-infected or uinfected PLC/PRF/5 cells for as long as 28 days in culture. In Table II the effect of two serum concentrations and insulin on antigen expression is shown. Low concentration of fetal calf serum as additive had a greater stimulatory effect on HAAg expression in infected cells than high serum concentration. When defined serum-free medium is supplemented with insulin (3×10^5 *M*), protein synthesis is largely increased, while viral antigen production is lowered, leading to an overall reduction of the relative amount of HAAg. The relative amount of HBsAg per milligram of extract of either infected or uninfected cells was elevated in cells grown in serum-containing medium, but reduced in medium supplemented only with insulin.

Serum-free medium supplemented with various concentrations of dexamethasone (DXM) and prostaglandin E_1 (PGE_1) were also tested. Glucocorticoid has been shown to increase the rate of synthesis of a number of proteins in hepatoma cells (9). By stimulation of special liver functions we tried to favor virus replication. Parallel cultures of infected PLC/PRF/5 cells were treated 8 days after infection for the following 9 days with various concentrations of DXM and PGE_1 in serum-free medium. As seen in Fig. 3, DXM stimulated HBsAg and HAAg production simultaneously in a concentration-dependent mode. A very similar response was observed for PGE_1. Se-

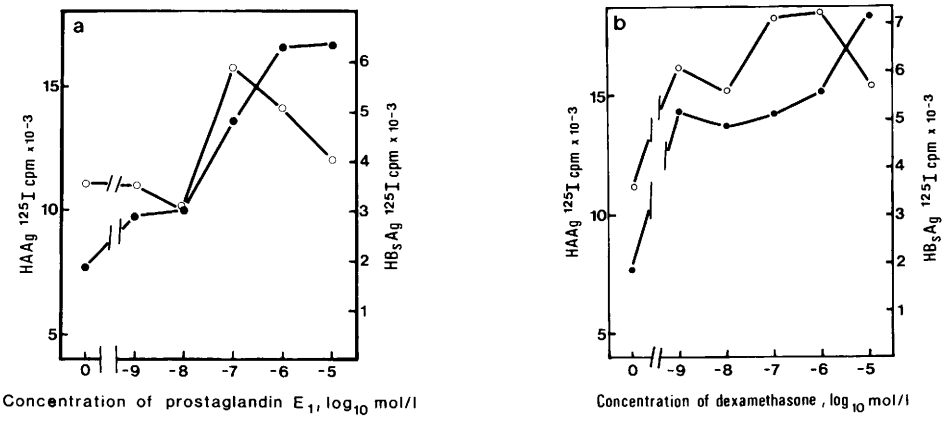

FIG. 3. Effect of prostaglandin (a) and dexamethasone (b) on expression of HBsAg (○) and HAAg (●) of HAV-infected cells. PLC/PRF/5 cells (triplicate flasks) were cultivated, beginning 8 days after infection with HAV, for the next consecutive 9 days with serum-free medium supplemented with the indicated concentrations of the hormones. Intracellular antigens were measured by RIA.

cretion of HBsAg into the extracellular space paralleled the amount of cell-bound antigen. Both hormones (at the highest concentration tested) seem to restore the stimulatory activity of medium supplemented with 2.5% FCS as compared with serum-free medium.

Discussion. HAV has now been classified as an enterovirus, but for a long time propagation of the virus in tissue culture was unsuccessful (1–6). One reason may be that, unlike other picornaviruses, HAV can only cause host cells to express antigens after a long lag period and it does not cause obvious cytopathology. As described before, serial passage of HAV in PLC/PRF/5 hepatoma cells reduces the lag phase between inoculation and first appearance of HAAg, and simultaneously the infectivity titer of HAV steadily increases to almost 10^8 $TCID_{50}$/ml after the 10th passage (11). From these preliminary observations, we concluded that HAV exerts no major effect on host-cell metabolism and this is corroborated by the present study.

The influence of HAV on host-cell metabolism was measured by the incorporation of radioisotope precursors into DNA and RNA. Our results show clearly that viral infection has no or at most little effect on overall macromolecule biosynthesis. Comparison of the growth kinetics of HAV-infected and uninfected cells indicate that HAV does not reduce the dividing capacity of host cells but lowers their plating efficiency. It is possible that this reflects a very limited cytopathic effect which does not become obvious in confluent cell layers. As a consequence of the noncytopathic growth of HAV, host-cell metabolism comes to a state of balance with virus replication. We have been able to keep persistently infected cells for more than 2 1/2 years in culture by serial passage of the cells.

The stability of the balance between host cell and virus was tested by altering cell culture conditions. The most dramatic effect was observed after change of the culture temperature. When HAV-infected PLC/PRF/5 cells were cultivated at 32°C, HAAg was detected only after a long lag period and in very small amounts, whereas at 37°C viral antigen was already found 10 days after infection and its rate of synthesis increased until it reached saturation about 30 days after infection. In earlier experiments, another line of PLC/PRF/5 cells was cultivated at 32°C after infection with HAV for optimal expression of HAAg (2). It appeared that at higher temperatures the survival of this line was impaired by mycoplasma infection. From temperature-shift experiments where antigen expression could be evoked at a higher temperature, we conclude that once the virus has penetrated the cell it can still survive under unfavorable conditions (low temperature) and be induced under better conditions. Whether we are dealing with a temperature-sensitive mutant or a variant of HAV must be clarified by further experiments. However, our observation with HAV-infected human embryo fibroblasts which express HAAg only at 32°C suggests that temperature dependence is a host-specific but not virus-specific characteristic (manuscript in preparation).

By altering culture factors, we hoped to learn more about host–virus interaction. Because of the growth characteristics of HAV in hepatoma cells, the various conditions (serum, insulin, dexamethasone, and prostaglandin E_1) had to be maintained for rather long (about 4 weeks), until viral antigen expression could be expected. The hormones were added freshly twice a week and the cells kept for 4 weeks. Despite the fact that some of the hormones have a rather short half-life *in vivo,* we still observed marked effects on cell–virus interaction. The well-known stimulatory effect of insulin on cell growth (10) was also observed in the hepatoma cells, but specific expression of HAAg and HBsAg was reduced. While insulin alone has no beneficial effect on specific production of HAAg or HBsAg, whole serum specifically stimulates the expression of HBsAg in uninfected and HAAg and HBsAg infected cells. Interestingly, low serum concentrations (0.5–2.5%) give higher yields than high concentrations.

Glucocorticoids have been described as having a beneficial effect on primary cultures of hepatocytes (12), as well as stimulating the replication of B-type retrovirus and polyoma virus in cell culture (13, 14). An enhancement of HBsAg secretion by PLC/PRF/5 cells within 24 hr has been reported (15). Over long periods of cultivation, an arrest of cell growth was observed. Our results not only confirm this but indicate that beside the stimulation of HBsAg secretion by dexamethasone, cellular

accumulation of HBsAg and HAAg is increased in a concentration-dependent mode. Similar results were observed with prostaglandin E_1.

In summary, it seems that the modulation of cell metabolism by hormonal factors (whole serum, insulin, glucocorticoid, and prostaglandin E_1) has a similar effect on expression of HBsAg and HAAg in PLC/PRF/5 cells, suggesting that similar or common regulatory mechanisms are involved. However, reduction of HBsAg production by HAV infection was very marked when cells were cultivated at different temperatures. In this case, the regulation of expression of either antigen seemed to be affected but with the opposite effect. In all cases, the total amount of HBsAg in infected cells is more or less reduced when compared to uninfected cells. It may be possible to correlate our results to conditions *in vivo;* comparable reports on patients who are chronic carriers of HBsAg and who had serological evidence of a simultaneous infection, indicated that HBsAg decreased at the acute stage of hepatitis A (16).

Under all cell culture conditions tested so far, only a relatively small change in HAAg expression was achieved, and no drastic shift from persistently to lytically infected cells was observed. The question whether inability of HAV to cause obvious cytolysis is a virus or a cell characteristic may be answered by studying infection and translation of viral RNA in different systems (in preparation). It is likely that mechanisms other than viral cytolysis *in vivo* cause the pathological changes observed in HAV-infected liver cells and cell-mediated immunity is a possible mechanism of hepatocellular necrosis during hepatitis A.

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