Persistent Infection of Human Fibroblasts by Hepatitis A Virus†

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SUMMARY

Infection of human embryo fibroblasts with hepatitis A virus (HAV), a picornavirus, leads to an inapparent, persistent infection; cultures can be passed serially with consistent recovery of the virus in the supernatant. All of the cells of a HAV carrier culture are infected and proliferate. Subcultivation under HAV-immune serum cannot achieve a cure or even a reduction in the number of infected cells in HAV carrier cultures. No interferon activity can be detected during HAV infection and persistence. Addition of exogenous interferon eliminates HAV infection in vitro. Persistence of HAV in vitro appears to contradict the clinical course of HAV infection in vivo. The system presented offers the possibility of evaluating the role of immunological injury of HAV-infected cells, an injury which may lead to damage of these cells and to elimination of HAV during an HAV infection in vivo.

INTRODUCTION

Hepatitis A virus (HAV), a picornavirus, is probably an enterovirus in terms of its biophysical and biochemical characteristics (Siegl & Frösner, 1978a, b; Coulepis et al., 1978), but its biological behaviour, in some respects, is quite different. During the last 3 years, HAV has been propagated in several cell systems (including a human hepatoma cell line) without causing any apparent cytopathic effect and the incubation period is extraordinarily long for a picornavirus. Provost & Hilleman (1979), Frösner et al. (1979), and Gauss-Müller et al. (1981) showed that HAV remained strictly cell-bound in diverse cell culture systems. Flehmig (1980, 1981) demonstrated release of HAV from infected cells, although no visible cellular damage was induced. It has been shown that HAV infection in cell cultures leads to a balance between cell metabolism and virus replication (Flehmig, 1981), although HAV does not seem to persist in vivo after an acute HAV infection.

In the present study, we describe a carrier state of human embryo fibroblasts infected with HAV. This investigation was undertaken to characterize the virus–cell relationship in vitro which maintains a balance between viral and cellular multiplication.

METHODS

Cell cultures. Lungs from human embryos 10 to 25 weeks old were used for production of human embryo fibroblast strains. Cell cultures were established by routine procedures using minimal essential medium, Hanks' salts + 10% foetal calf serum as growth medium. Cultures were subpassed once weekly by 1:2 dilution. HAV carrier cultures (human embryo fibroblasts persistently infected with hepatitis A virus) were held under the same conditions as the uninfected parent strains.

Hepatitis A virus. Hepatitis A virus (HAV) has been adapted to human embryo fibroblasts as described previously (Flehmig et al., 1981). The supernatant of the second passage of NAV in fibroblasts was prepared as stock culture virus and kept at −80°C.

Radioimmunoassay for detection of HAV. Detection of HAV by radioimmunoassay (RIA) was performed as described previously (Flehmig, 1980).

Supernatant from uninfected control fibroblasts was taken as a negative control. Radioactivity (ct/min) of four parallel antigen samples minus ct/min of the mean value of eight negative controls is plotted in the figures.

† Dedicated to Professor Wilhelm Seyffert on the occasion of his 60th birthday.
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Samples were considered positive if the ratio of the ct/min of the sample divided by the ct/min of the negative control (P/N ratio) was greater than 2.1. A P/N ratio of 2:1 was achieved if the difference between negative control and sample was greater than 300 to 400 ct/min.

**Immunofluorescence microscopy.** Detection of HAV in human embryo fibroblasts by indirect immunofluorescence microscopy (IF) has been described in detail (Flehmig, 1980).

**Determination of infectious HAV particles.** The supernatant from HAV-infected cell cultures was centrifuged and filtered to remove cells. Eight microplate wells containing human embryo fibroblasts were inoculated with 150 μl of each virus dilution in minimal essential medium containing Earle's salts + 2% calf serum and the cultures were kept at 37°C in a 5% CO2 atmosphere for 18 days. Cultures were then frozen and thawed three times and the supernatants used in a RIA for the detection of HAV antigen. Titres are expressed as TCID50 calculated by the Kärber method (Kärber, 1931).

**Harvest of cell-bound HAV.** The medium of cell cultures was removed and 1 ml Hanks' balanced salt solution was added to each tube. Cells were then frozen and thawed three times and the supernatant cleared by centrifugation for 30 min at 3000 g. The supernatant was used in a RIA for the detection of HAV antigen or was titrated for infectious HAV particles as described above.

**Interferon assay.** Interferon (IFN) titrations were performed by plaque reduction assay as described previously (Vallbracht et al., 1981). IFN titres are expressed in terms of reference standard G-023-902-527 (HuIFN-β) from the NIH, Bethesda, U.S.A.

**Interferon and antiserum.** Human fibroblast interferon (HuIFN-β) was obtained from Rentschler Co. (Laupheim, F.R.G.) and antiserum against HuIFN-β from a patient treated with HuIFN-β (Vallbracht et al., 1981).

**RESULTS**

**Establishment of a carrier culture with hepatitis A virus**

Diploid human embryo fibroblasts were inoculated with HAV at a m.o.i. of 0.1 and were kept at 37°C for 4 weeks. At the intervals indicated in Fig. 1, the supernatant was tested for HAV by a RIA. Simultaneously inoculated fibroblast cultures on coverslips were stained by an indirect IF technique for the detection of cell-bound HAV. HAV-positive cells were first detected by IF after 4 days, the proportion rising to 100% after 10 days. HAV antigen appeared in the supernatant 13 days after infection.

Four weeks after infection, cultures were subpassed as described. From then on, the infected cultures could be maintained according to the same growth schedules as employed for the uninfected parent cells. This procedure resulted in the establishment of an HAV carrier culture which was morphologically indistinguishable from normal control fibroblasts. In the course of HAV infection of human fibroblasts no cytopathic effect could be detected, even after multiplicities of 1, 10 and more. Persistent infection of human fibroblasts with HAV could also be achieved at these multiplicities at 37°C as well as 34°C incubation temperature (not shown).

HAV carrier fibroblasts could also be maintained with a reduced amount (5 to 2%) or even without foetal calf serum. Over a period of 7 weeks, HAV carrier fibroblasts could be kept under these conditions at 37°C or 34°C without medium change. This procedure resulted only in a slight increase of non-viable cells in the HAV carrier cultures compared to normal fibroblasts (not shown). After storage at −80°C HAV carrier fibroblasts could be subpassed like control cultures.

**Determination of intracellular and extracellular HAV**

In further experiments cultures were subpassed weekly by 1:2 dilution and growth medium was removed for quantitative assays. Fig. 2 shows the results of continual monitoring of the release of HAV antigen and infectious particles of HAV carrier cultures over a period of 20 passages. The amount of infectious HAV in the supernatant of HAV carrier cultures ranged between 10⁶ and 10⁷ TCID50/ml in the absence of any recognizable cytopathic effect. Seven days after subpassage each passage was examined by IF. Over the whole period of 20 passages, 100% of cells contained specific HAV antigen as indicated in Fig. 2.

**Enumeration of cells containing HAV antigen**

Quantitative microscopic observations made on replicating HAV carrier cultures 1 to 48 h after 1:2 dilution onto coverslips showed that as early as 1 h after plating, all the fixed cells
Hepatitis A virus persistence

Fig. 1. Propagation of HAV in human embryo fibroblasts. Determination of HAV antigen by RIA (---) and of HuIFN-β (-----) in the supernatant of infected cells is shown.

contained HAV antigen. Proliferation of HAV-infected cells was also indicated by immuno-
fluorescence of cells in mitosis in HAV carrier cultures.

Growth rates of carrier cells

It was mentioned that the persistently infected HAV carrier fibroblasts could be maintained under the same conditions as the uninfected parental fibroblasts. In order to determine the generation time, growth rates from HAV carrier cultures and normal fibroblasts were established. Cultures were trypsinized and resuspended in fresh growth medium at a concentra-
tion of 1 x 10^5 cells/ml. Tubes were seeded with 1 ml of cell suspension. At various intervals thereafter, trypan blue was added and the viable cells of two tubes of each group were counted separately. Fig. 3 shows that HAV carrier fibroblasts have a slightly lower seeding efficiency and at least initially a slightly decreased growth rate in comparison to uninfected fibroblasts; this was reproduced in three experiments.

Interference studies

As demonstrated in Fig. 1 and 2 no interferon activity could be demonstrated during passages of HAV carrier fibroblasts. To exclude a possible role of undetectable amounts of interferon in the maintenance of HAV carrier cells, antibodies against human fibroblast interferon (anti-
HuIFN-β) were added in a concentration neutralizing 10^3 IU/ml HuIFN-β. Subcultivation of HAV carrier fibroblasts in the presence of anti-HuIFN-β did not result in any cytopathic effect.

Next, we examined the susceptibility of HAV carrier cultures to cytopathic viruses, in order to investigate further interference mechanisms of HAV carrier cultures with other virus strains, especially enteroviruses. HAV carrier fibroblasts showed no resistance to the virus strains tested. Titration of herpesvirus hominis types 1 and 2, vesicular stomatitis virus and some enterovirus strains, poliovirus types 1, 2 and 3, coxsackieviruses A9, B1, B2, B3, B5 and echovirus 9, in microplate cultures of normal and HAV carrier fibroblasts resulted in comparable titres. Further experiments showed also that the kinetics of propagation of poliovirus type 2 in normal and HAV carrier fibroblasts were not significantly different.

Effect of anti-HAV immune serum on HAV carrier fibroblasts

In order to exclude the presence of virus inhibitors in the foetal calf serum used, undiluted serum was mixed with undiluted HAV, the mixture was allowed to stand for 3 h at room
temperature, then titrated in human fibroblasts and compared with a control mixture of Hanks’ salt solution and HAV. None of the foetal calf sera used contained demonstrable antiviral substances.

In the following experiments human anti-HAV immune serum was added at a concentration of $10^5$ to HAV carrier cultures. Fifteen subpassages of HAV carrier cultures in the presence of this immune serum were monitored for the release of HAV and the number of cells containing viral antigens. As demonstrated in Fig. 4, the immune serum used contained sufficient anti-HAV antibodies to keep the medium free of infectious virus and only a slight amount of non-infectious HAV antigen could be detected by RIA.

Examination of coverslip cultures prepared during subcultivation by indirect IF staining revealed no decrease in the proportion of HAV-positive cells. Carrier cultures returned to antibody-free medium released equal amounts of HAV into the supernatant as did untreated control HAV carrier cultures (not shown).

Even further subcultivation in the presence of anti-HAV immune serum could not achieve ‘curing’ of HAV carrier fibroblasts.

Effect of human fibroblast interferon on HAV carrier fibroblasts

Fig. 5 shows the effect of exogenous fibroblast interferon (HuIFN-β) on HAV carrier fibroblasts. Cultures were passed weekly with growth medium containing $10^3$ IU/ml HuIFN-β and monitored for the release of HAV and the number of cells expressing HAV antigen, as compared to untreated control HAV carrier cells. After four passages under interferon,
Continual monitoring of HAV carrier fibroblasts over 15 passages in the presence of HAV immune serum (——) or anti-HAV-negative preimmune serum from the same patient (-----). Specific HAV antigen (ct/min) and infectious HAV (TCID₅₀) were determined in the supernatant of HAV carrier cells.

Continual monitoring of HAV carrier fibroblasts over 10 passages in the presence of HuIFN-β (——) in comparison to untreated HAV carrier fibroblasts (-----). HuIFN-β was added once a week to a final concentration of 10⁵ IU/ml. Specific HAV antigen (ct/min) and infectious HAV (TCID₅₀) were determined in the supernatant of HAV carrier cells.

### Table 1. Effect of human fibroblast interferon on HAV carrier fibroblasts

<table>
<thead>
<tr>
<th>No. of HAV carrier passages with 10⁵ IU/ml HuIFN-β</th>
<th>Infectious cell-free HAV</th>
<th>Infectious cell-bound HAV</th>
<th>HAV antigen (detected by RIA)</th>
<th>HAV antigen (detected by IF)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>NT*</td>
<td>1</td>
<td>1</td>
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<td>NT</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>5-10</td>
<td>20 negative</td>
<td>20 negative</td>
<td>20 negative</td>
<td>20 negative</td>
</tr>
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</table>

* NT, Not tested.

Infectious HAV could no longer be detected and no specific HAV antigen appeared in the supernatant of HAV carrier fibroblasts. Indirect IF staining of coverslip cultures prepared during subcultivation in the presence of interferon revealed a stepwise decrease in the proportion of HAV-positive cells.

During the subcultivation of HAV carrier fibroblasts with interferon, half of the cells of each passage were returned to an interferon-free medium and then subpassed without interferon. Table 1 summarizes the effect of exogenously added HuIFN-β on HAV carrier fibroblasts. Five
passages with $10^3$ IU/ml HuIFN-β resulted in the elimination of the HAV infection. Twenty subsequent passages in an interferon-free medium confirmed the disappearance of all HAV markers in the interferon-treated cultures. Interferon-cured HAV carrier cells could be re-infected with HAV and subpassed further as HAV carrier cells (not shown). The elimination of HAV infection could also be achieved with $10^3$ IU/ml of human leukocyte interferon (HuIFN-α) (not shown).

**DISCUSSION**

The data presented here show that the infection of human fibroblasts with HAV results in a persistent infection which leads to a balance between cell metabolism and virus replication. Similar results concerning the establishment of a persistent HAV infection in a foetal rhesus kidney cell line have been described by Flehmig (1981).

HAV has been identified as a picornavirus, probably belonging to the enterovirus group (Siegl & Frösner, 1978a, b) although the biological behaviour of HAV in cell cultures seems to be quite unique among these viruses. Establishment of a persistent enterovirus infection in cell cultures is not common. Ackermann & Kurtz (1955) observed that, under suitable circumstances, poliovirus can persist in a culture of HeLa cells while the cells are undergoing prolonged and extensive multiplication. In this system, persistent infection of poliovirus was dependent on the presence of immune serum in the culture fluid. Persistent infection by coxsackievirus A9 in HeLa cells has been described by Takemoto & Habel (1959). This virus–cell relationship appeared to be similar to that reported for poliovirus carrier cultures, except that antiviral antibody was not necessary for the maintenance of the carrier state. Only a small proportion of the cells in carrier cultures were infected, and immune serum eliminated virus from the culture.

These enterovirus carrier systems appear to be quite different from the carrier state of HAV. As demonstrated in this study, no changes in the usual cultural procedures were required to maintain carrier systems. Immunofluorescence studies revealed that, after the establishment of this carrier system, all the cells were infected with HAV. Subpassages in the presence of anti-HAV serum did not decrease the proportion of HAV-positive cells, although no infectious virus could be detected in the supernatant. These results, plus the fact that cultures in which 100% of the cells contain HAV antigen can still multiply at a rate close to that of uninfected fibroblasts, prove that cells can divide repeatedly and that the antigen-producing potential can be passed from a parent cell to daughter cells.

Interferon does not seem to play an important role in the maintenance of a HAV carrier state. We were unable to detect interferon in the supernatant during the infection or the subpassages of HAV carrier fibroblasts. Furthermore, addition of antibodies against human fibroblast interferon did not induce a cytopathic effect in these carrier systems such as was described recently for measles virus persistence in lymphocytes (Jacobson & McFarland, 1982). On the contrary, exogenous interferon led to the elimination of the infection in HAV carrier cells.

Our study failed to show any intracellular interference between HAV infection and several cytopathic viruses. Viral-attachment interference between HAV carrier cells and cytopathic enterovirus strains could also not be demonstrated.

In summary, our results did not indicate any significant difference between HAV-infected cell cultures and the uninfected parental fibroblasts, although a large number of infectious HAV particles were produced by and released from the carrier cells. The mechanism which induces this balanced relationship between virus propagation and cell proliferation remains unknown. A role of defective interfering particles or ts mutants in the maintenance of persistent infection, as has been shown in several systems (De & Nayak, 1980; Nishiyama, 1977; Holland et al., 1976; Youngner & Quagliana, 1976; Preble & Youngner, 1975), was not apparent in our study. However, no comparable studies are possible at present, because there is no cell culture system in which HAV induces a cytopathic effect.

The purpose of this study was not the definitive clarification of the mechanism by which HAV induces a persistent infection but rather the demonstration of this phenomenon *in vitro*. There appears to be a discrepancy between persistent HAV infection *in vitro* and the course of HAV infection *in vivo*. 
Hepatitis A virus does not seem to play a role in the development of persistent hepatitis in vivo. Therefore, the data on HAV persistence in vitro are not easily reconciled with the clinical course of hepatitis A infection. As far as we know, HAV never produces chronic infections in man and one possible mechanism for the elimination of HAV in vivo could be the induction of interferon. Our results, however, may also be compatible with the hypothesis that the symptoms of hepatitis A are not a consequence of the cytocidal infection of hepatic cells but rather of the elimination of infected cells by immunological reactions. The system we present of HAV carrier cells offers the possibility of clarifying the role of cell- and antibody-mediated cytotoxicity during the course of an acute HAV infection in vivo.

REFERENCES


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