Induction of Hepatitis B Surface Antigen in Human Hepatoma-derived Cell Lines

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SUMMARY

Cultures of two human hepatoma cell lines were examined for expression of hepatitis B virus surface antigen (HBsAg). The PLC/PRF/5 cells secreted HBsAg continuously into the culture medium, whereas Mahlavu cells did not secrete the antigen. However, cytoplasmic antigen was detected in a low percentage (<5%) of the Mahlavu cells. The expression of HBsAg also was assayed in cultures treated with dexamethasone (DXM), 5-iodo-2'-deoxyuridine (IdUrd), or both. The results demonstrated that: (i) DXM stimulated secretion of HBsAg by PLC/PRF/5 cells but not by Mahlavu cells; (ii) the percentage of Mahlavu cells expressing cytoplasmic HBsAg was not increased in any cultures if the medium was replaced at 24 to 48 h intervals but was increased approx. fivefold within 4 days in cultures treated with DXM or IdUrd/DXM if the medium was not changed. However, no increase was noted in the intensity of the immunoperoxidase stain of PLC/PRF/5 cells that expressed cytoplasmic antigen in any DXM cultures; (iii) HBsAg expression was stimulated to a lesser extent in IdUrd/DXM cultures than in DXM cultures and was not enhanced in IdUrd cultures. Thus, DXM enhanced secretion of HBsAg by PLC/PRF/5 cells within 24 h and, after a delay, enhanced expression of cytoplasmic antigen by Mahlavu cells. However, antigen secretion by Mahlavu cells evidently was blocked.

INTRODUCTION

Hepatitis B virus (HBV) has been associated with a high incidence of human hepatoma in diverse geographic areas (Prince et al., 1975; Reys et al., 1976; Tabor et al., 1977; Melnick, 1978; Szmuness, 1978), and HBV antigens have been detected in hepatoma tissue specimens (Nayak et al., 1977; Thung et al., 1979). Several cell lines that differ in the expression of HBV gene products have been established from these tumours (Prozesky et al., 1973; Alexander et al., 1976, 1978; Macnab et al., 1976; Aden et al., 1979). For example, hepatitis B surface antigen (HBsAg) has not been detected in cultures of the Mahlavu cell line (Prozesky et al., 1973), whereas cells from the PLC/PRF/5 line, established by Dr J. J. Alexander, secrete HBsAg into the culture medium (Macnab et al., 1976; Alexander et al., 1976, 1978). The antigen was concentrated and purified from the culture fluids and was viewed by electron microscopy (Macnab et al., 1976). The antigen produced in vitro is reactive in commercial radioimmunoassay (RIA) for HBsAg and is retained by specific anti-HBs immunosorbents. Furthermore, the major polypeptides associated with purified HBsAg from sero-positive plasma are detectable in purified antigen from culture fluids (Monjardino & Crawford, 1979;
Marion et al., 1979; Skelly et al., 1979). These results demonstrate the immunological and structural similarities of HBsAg produced in vivo and in vitro.

There are numerous reports of chemical stimulation of virus production or expression in cell cultures. The incubation of various murine cells in medium containing 5-iodo-2'-deoxyuridine (IdUrd) or 5-bromo-2'-deoxyuridine induces production of type-C retrovirions (Aaronson et al., 1971; Lowy et al., 1971). The replication of type-B virions by cultured mouse mammary tumour cells is stimulated by dexamethasone (DXM), a glucocorticosteroid, in the culture medium (Parks et al., 1974; Scolnick et al., 1976), and treatment of cells with IdUrd and DXM enhances production of both virus types (Fine et al., 1974; Wivel & Yang, 1976; Wu et al., 1976). In attempts to stimulate HBsAg production, we treated cultures of Mahlavu and PLC/PRF/5 cells with IdUrd, DXM, or both. The halogenated pyrimidine did not enhance antigen production, but DXM stimulated the secretion of antigen by PLC/PRF/5 cells and enhanced cytoplasmic expression of the antigen by Mahlavu cells.

METHODS

Cell cultures. Two human hepatoma-derived cell lines, PLC/PRF/5 and Mahlavu, were kindly provided by Dr J. J. Alexander. The PLC/PRF/5 cell line produces HBsAg, while the Mahlavu cell line has not been shown to secrete HBsAg (Alexander et al., 1978). The PLC/PRF/5 cells used for these experiments were between passages 30 and 40. The Mahlavu cells were approximated to be near passage 120. Both of these cell lines are epithelial in nature and grow in monolayers. A cervical carcinoma cell line (CC22) was used as an HBV-negative control (Melnick et al., 1979). This epithelial cell line was used at passage number 50.

Treatment of cell cultures. The growth medium was Autopow (Flow Laboratories) supplemented with 10% foetal bovine serum (Gibco), 250 µg/ml penicillin and 180 µg/ml streptomycin, 0.03% L-glutamine, 0.075% sodium bicarbonate and 1 × non-essential amino acids (Gibco). Viable cells (determined by trypan blue exclusion) were seeded in 60 mm tissue culture dishes (Lux, Newbury Park, Calif., U.S.A.) at a density of 1 × 10⁶ cells/dish in 5 ml medium and were grown in a 5% CO₂ atmosphere at 37 °C. For the immunocytochemical studies, cells were seeded on 15 mm glass coverslips and grown under the conditions described.

For induction, cell cultures were treated as described by Bronson et al. (1978). At 24 h post-seeding (day 1), the medium was replaced with either fresh medium (untreated cultures) or with medium containing either 100 µg IdUrd/ml (Sigma; IdUrd cultures), 1 × 10⁻⁶ M-DXM (Sigma; DXM cultures), or a combination of IdUrd and DXM at these concentrations (IdUrd/DXM cultures). Previous experiments, which tested increasing concentrations of DXM, had shown that a level of 1 × 10⁻⁶ M was the optimum for induction of HBsAg in the PLC/PRF/5 cells. After incubation for 24 h (day 2), and again on days 4, 5 and 7, the medium on DXM and IdUrd/DXM cultures was replaced with medium containing 1 × 10⁻⁶ M-DXM, and medium on the IdUrd cultures was replaced with growth medium. Culture fluids were frozen at −70 °C for later testing for HBsAg. In a second series of experiments, the procedure was identical except that the medium was not changed after day 5.

Six coverslips were removed from each set of cultures on days 5, 7 and 9, washed with phosphate-buffered saline (PBS) for 5 min at room temperature, air-dried, fixed in acetone for 10 min at 4 °C, and stored in a vacuum desiccator at 4 °C. At each time interval, fluids from each culture were stored at −70 °C.

Preparation of antisera and immunoconjugates. Anti-HBs serum was prepared by immunization of New Zealand white rabbits with purified HBsAg/adw (Dreesman et al., 1972a). Before use, the antiserum was adsorbed with glutaraldehyde-cross-linked normal
human serum proteins and with a powder prepared from an acetone-extracted dog liver. Hepatitis B core antigen (HBcAg) was derived from an HBV-infected chimpanzee liver and inoculated into rabbits as described by this laboratory (Fields et al., 1975/76; L. M. Schulster & G. R. Dreesman, personal communication). The specificity of each reagent was confirmed by immunodiffusion and immunoelectrophoresis against normal human serum and by radioimmunoassay employing Austria-II and Ausab test kits (Abbott Laboratories, North Chicago, Ill., U.S.A.) In addition, it was demonstrated that the anti-HBs reactivity was removed after adsorption of the serum with a glutaraldehyde-insolubilized γ-globulin fraction of an HBsAg-positive plasma (Avrameas & Ternynck, 1969). The immunoreagents also were tested for antibody specificity on sections of a paraffin-embedded HBV-positive liver from a patient with chronic antigenaemia (Cabral et al., 1978). Goat anti-rabbit IgG serum was obtained commercially (Immuno-Reagents Associates, Seguin, Tex., U.S.A.).

Antiserum to horseradish peroxidase (anti-HRPO; Sigma) was prepared by intramuscular injection of rabbits on days 0, 14, and 28 with 0.1 mg of isoelectrophoretically purified HRPO emulsified in Freund's complete adjuvant. The animals were bled by day 35. Rabbit peroxidase/anti-peroxidase (PAP) reagent was prepared as described by Sternberger (1973). Briefly, the equivalence zone of a rabbit anti-HRPO serum was determined with a qualitative immunoprecipitation test. A quantity of HRPO, selected to give 1.25 times the antigen-equivalence concentration (8.75 mg), was mixed with 20 ml anti-HRPO serum and stirred for 1 h at room temperature. The precipitate was pelleted and washed three times with 0.15 M-NaCl at 4 °C. The final precipitate was resuspended in 16 ml of a solution containing 8.75 mg HRPO/ml in 0.15 M-NaCl, adjusted to pH 2.3 with 1 M-HCl, and neutralized quickly (pH 7) with 0.1 M-NaOH. A solution of 0.08 M-sodium acetate and 0.15 M-ammonium acetate (1.6 ml) was added to the reaction mixture, which was then centrifuged at 17500 rev/min at 4 °C. An equal volume of saturated (NH₄)₂SO₄ was added to the supernatant fluid with constant mixing at 4 °C. After low-speed centrifugation at 4 °C, the precipitate was washed once with 50% (NH₄)₂SO₄, dissolved in distilled H₂O and subsequently dialysed against several changes of buffer (0.08 M-sodium acetate and 0.15 M-ammonium acetate). The molar ratio of HRPO to anti-HRPO IgG in the final conjugate ranged from 1.5 to 2.

Antigen detection techniques. Acetone-fixed cells were studied for expression of HBV-specific antigens (HBsAg and HBcAg) by the PAP method (Sternberger, 1973). In confirmation of a previous report (Alexander et al., 1978), we noted that the reactivity of intracellular HBsAg was diminished greatly if PLC/PRF/5 cells were fixed with ethanol. Briefly, the procedure was performed as follows. The coverslips first were incubated in PBS containing 10% (v/v) normal goat serum (Immuno-Reagents Associates). Rabbit antisera, specific for either HBsAg or HBcAg, were each diluted 1:4 and used as the primary antibody. After a 30 min incubation, the coverslips were washed and then treated with goat anti-rabbit IgG (1:64). The PAP reagent was used at the optimum dilution of 1:10 during the final incubation period. Each antiserum and the PAP reagent were diluted in 10% normal goat serum in PBS and filtered through a 0.45 μm filter (Millipore) before use. Between each of the antiserum and PAP reagent incubation periods, the coverslips were washed four times (7 min/wash) with 2% normal goat serum in PBS. Each of the reactions was done by incubation for 30 min at room temperature in a humidified chamber. After the coverslips had been treated with the PAP reagent, they were washed three times (10 min/wash) with 0.05 M-tris-HCl buffer pH 7.6 (tris buffer), without serum. The washed coverslips were then incubated for 8 min in the dark with Hanker–Yates reagent (Polysciences, Warrington, Pa., U.S.A.) prepared at a concentration of 1 mg/ml in tris buffer containing 0.01% H₂O₂ and filtered (0.45 μm). Hanker–Yates reagent is composed of a mixture of p-phenylenediamine and pyrocatechol, which is converted into an osmiophilic co-polymer by the HRPO oxidation reaction (Hanker et al., 1977). The coverslips were washed in distilled water for 2 min,
dehydrated with graded ethyl alcohol and toluene, and mounted with Permount (Fisher Scientific, Houston, Tex., U.S.A.).

Medium harvested from each of the cell cultures at the time intervals listed above was assayed for HBsAg by RIA (Ausria-II, Abbott Laboratories). Each supernatant fluid was tested undiluted or, where warranted, at a 1:15 dilution, and the level of HBsAg protein was determined from a standard curve established with purified preparations of HBsAg/adw with a predetermined protein concentration (Dreesman et al., 1972b). Because variations have been noted among lots of RIA kits, a standard curve was established for each kit.

The data presented in Fig. 2 were analysed using the following formula, in which each single value was compared to the mean of the sample (Sokal & Rohlf, 1969):

\[
t_s = \frac{Y_1 - \bar{Y}_2}{S_2 \sqrt{n_2 + 1}}, \text{ degrees of freedom } = n - 1,
\]

where \(Y_1\) = data point being tested for acceptance or rejection; \(Y_2\) = sample mean of values without the \(Y_1\) value; \(S_2\) = standard deviation of the sample; and \(n_2\) = sample size (excluding the \(Y_1\) value).

RESULTS

Assay of tissue culture fluids for HBsAg

The amount of HBsAg in supernatant fluids from each set of cultures was correlated with cell density, culture conditions (i.e. with or without induction) and length of incubation. At day 4, the amount of antigen secreted by the DXM cultures of PLC/PRF/5 cells was approximately twice that of untreated cells (Fig. 1a). However, by day 7, the concentration of HBsAg in supernatant fluids from both cultures was similar. No increase in HBsAg production was noted in IdUrd cultures, and the amount secreted by IdUrd/DXM cultures was intermediate between that of DXM cultures and untreated cultures (Fig. 1a). At each time interval, there was an increase in the number of cells in untreated cultures, whereas no significant increase in cell number was observed in any of the treated cultures (Fig. 1b).

A comparison of the quantities of HBsAg produced/10^6 cells by the four sets of cultures is shown in Fig. 2. The amount of antigen produced in untreated and IdUrd cultures did not change significantly within the 9 day period. However, there was a marked increase (roughly threefold) in the secretion of HBsAg by the DXM cultures, with maximum constant stimulation observed on days 5, 7 and 9. The differences between untreated and DXM cultures were significant \((P < 0.01, \text{ unpaired Student } t\)-test). While IdUrd/DXM cultures did not show an increase in HBsAg levels on days 2 and 4, significant induction did occur on days 5, 7 \((P < 0.01)\) and 9 \((P < 0.02)\).

No HBsAg was detected in the supernatant fluids from any of the treated Mahlavu cell cultures.

Assay of tissue culture cells for HBsAg

Immunoperoxidase staining of untreated cultures of PLC/PRF/5 cells for HBsAg exhibited a weak, general cytoplasmic reaction with rare foci of more intensely stained cells (Fig. 3b). However, no marked differences were observed between the untreated and treated sets of cultures with this technique (compare with Fig. 3c). The specificity of these assays for HBsAg in these experiments was demonstrated in that (i) no reaction was noted when normal rabbit serum was used as a primary serum (Fig. 3a); (ii) adsorption of the primary anti-HBs serum with insolubilized sero-positive HBsAg plasma blocked the reaction; and (iii) no specific reaction was noted when CC22 cells were incubated with a primary anti-HBs serum.
Induction of HBsAg in hepatoma cells

Fig. 1. Kinetic analysis of HBsAg production and cell multiplication in the PLC/PRF/5 line of human hepatoma cells with and without chemical induction. Twenty-four h after seeding of $1 \times 10^6$ cells/tissue culture dish (large arrow), the culture fluids were replaced with fresh growth medium (untreated cultures, ○) or with medium containing 100 μg/ml IdUrd (IdUrd cultures, ●), $1 \times 10^{-4}$ M-DXM (DXM cultures, □), or both (IdUrd/DXM cultures, ■). On the days indicated (small arrows), supernatant fluids of the untreated and IdUrd cultures were replaced with fresh growth medium, and the supernatant fluids of the cultures treated with DXM or IdUrd/DXM were replaced with medium containing $1 \times 10^{-6}$ M-DXM. The supernatant fluids removed from four individual tissue culture dishes at each time interval were monitored for production of HBsAg (solid-phase RIA) (a). Total cell counts (trypan blue exclusion) were also performed on cultures (b).

Fig. 2. Amount of HBsAg secreted daily per $10^6$ PLC/PRF/5 cells grown in the presence or absence of chemical inducers. The induction protocol is described in the legend to Fig. 1. Culture fluids (1:15 dilution) from four culture dishes at each time were tested for HBsAg (solid-phase RIA). Quantities of HBsAg were determined from standard curves generated with known concentrations of purified HBsAg/adw. Vertical bars represent one standard deviation (Student t-test). Symbols are as for Fig. 1.

An unexpected finding was the cytoplasmic staining for HBsAg in a low percentage (<5%) of cells in treated (Fig. 3f) and untreated (Fig. 3e) Mahlavu cultures when the medium was replaced at the intervals indicated in Fig. 1 and 2. In contrast, by day 9 the number of intensely positive-staining cells increased in the DXM or IdUrd/DXM cultures in which the medium was not replaced after day 5 (Fig. 4a). The untreated Mahlavu cultures did not exhibit an increase in intracellular HBsAg production under conditions where the media were left unchanged after day 5. The percentage of cells producing HBsAg was quantified by counting a minimum of 500 cells on several coverslips from cell cultures grown in the presence or absence of inducers. The results are summarized in Table 1. There was an increase from the 3 to 4% positive-staining cells observed in the untreated and IdUrd-treated cultures to 20 to 30% seen in the DXM and IdUrd/DXM-treated coverslips.

These immunohistocytochemical observations were verified with the following experiment. Acetone-fixed coverslips of untreated and DXM-treated Mahlavu cultures were incubated for 2 h at 39°C with 0.1 ml of the iodinated human anti-HBs obtained from a commercial RIA kit (Austria-II). A culture of CC22 cells (Melnick et al., 1979) was utilized as an HBV-negative control. The results given in Table 2 show an increase in the quantity of
Fig. 3. Peroxidase/anti-peroxidase (PAP) assay of HBsAg expressed in PLC/PRF/5 and Mahlavu human hepatoma-derived cell lines before and after induction with DXM. (a, b) Micrographs of untreated cultures of PLC/PRF/5 cells; (c) DXM-treated PLC/PRF/5 cells; (d, e) micrographs of untreated Mahlavu cells; (f) DXM-treated Mahlavu cells. The conditions are the same as given in the legend to Fig. 1. All micrographs were made from cells on day 9. The primary serum used in the PAP staining of cells shown in (a) and (d) was normal rabbit serum (negative control), whereas a rabbit anti-HBs serum was used as the primary serum on the cells in (b), (c), (e) and (f).
Induction of HBsAg in hepatoma cells

Fig. 4. PAP staining for HBsAg expression by (a) Mahlavu cells and (b) PLC/PRF/5 cells after induction with $1 \times 10^{-6}$ M-DXM. After induction the cells were held without changing the medium from days 5 to 9 in culture fluid containing $1 \times 10^{-6}$ M-DXM. A rabbit anti-HBs serum was used as the primary antiserum in the PAP staining of these cells.

Table 1. Quantification of untreated or inducer-treated Mahlavu cell cultures for immunoperoxidase-positive staining for HBsAg

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Positive</th>
<th>Negative</th>
<th>Positive cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>21</td>
<td>479</td>
<td>4.2</td>
</tr>
<tr>
<td>IdUrd</td>
<td>19</td>
<td>481</td>
<td>3.8</td>
</tr>
<tr>
<td>DXM</td>
<td>106</td>
<td>394</td>
<td>21.2</td>
</tr>
<tr>
<td>IdUrd/DXM</td>
<td>131</td>
<td>376</td>
<td>26.2</td>
</tr>
</tbody>
</table>

Table 2. Reactivity of iodinated human anti-HBs* with HBV-negative (N) and HBV-positive (P) cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>P/N ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC22 cells†</td>
<td>1.00</td>
</tr>
<tr>
<td>Mahlavu, untreated</td>
<td>1.38</td>
</tr>
<tr>
<td>Mahlavu, DXM-treated</td>
<td>2.32</td>
</tr>
</tbody>
</table>

* Antibody was obtained from a commercial RIA kit (Ausria II).
† An epithelial cell line derived from a human cervical carcinoma (Melnick et al., 1979) which was used as a negative control (N).

HBsAg in the DXM-treated cells over that observed for untreated cells. However, there was no evidence of the secretion of HBsAg into the supernatant fluid of any treated or untreated Mahlavu cultures, as determined by RIA.

Two additional experiments demonstrated that (i) cultures of PLC/PRF/5 cells in which medium was not changed after day 5 failed to display an enhanced expression of HBsAg (Fig. 4b) and (ii) HBCAg was not detected in any cultures of either cell line.

Discussion

The results of the RIA and PAP assays confirmed an earlier report (Aden et al., 1979) that the PLC/PRF/5 cells produce and secrete HBsAg continuously. In agreement with the immunofluorescence results by Desmyter et al. (1978), the antigen was found in the cytoplasm. Our data also demonstrated that antigen secretion/cell increased significantly in cultures treated with DXM. Secretion of HBsAg/cell was not enhanced in IdUrd cultures and
was stimulated in IdUrd/DXM cultures by approx. 50% of the antigen level in DXM cultures. Under the experimental conditions described here, DXM induced HBsAg secretion, but IdUrd did not and, in fact, it depressed the stimulation exerted by DXM.

We also confirmed a report (Prozesky et al., 1973) that the Mahlavu cells do not secrete HBsAg, although intracellular antigen was detected in a few cells. When the medium was replaced at 24 to 48 h intervals, induction did not increase the number of cells expressing intracellular antigen, but when the medium was not replaced for at least 4 days, DXM increased by five- to tenfold the number of cells expressing intracellular antigen by day 9. However, even in these cultures, no extracellular antigen was detected. Thus, it appears that DXM either does not stimulate secretion of the antigen or does not stimulate intracellular production to a level that allows secretion, although it is possible that the amount of antigen secreted is too small (less than 1 ng/ml) to be detected by RIA.

A similar delay in production of HBsAg by Hep3B human hepatoma cells was reported by Aden et al. (1979), who noted that daily medium changes resulted in very low levels of the antigen only after incubation for at least 8 days, whereas antigen was expressed within 6 days in cultures incubated in conditioned medium. Those authors suggested that the most plausible explanations for the delay were: (i) antigen is produced only by cells in a resting phase resulting from incubation in spent medium, or (ii) antigen-producing cells are those that have matured to the terminal differentiation stage.

Thus, our results indicate that two human hepatoma cell lines produce HBsAg but differ in that the continuous secretion of the antigen by PLC/PRF/5 cells is enhanced by DXM, whereas there is a delayed production, but no secretion, of the antigen by Mahlavu cells in DXM-treated cultures. If correlated to the results of Aden et al. (1979), in which there is a delayed secretion of HBsAg by Hep3B cells, it is apparent that different mechanisms control production and, possibly, secretion of HBsAg by these three lines. The different mechanisms might involve the following: (i) the number of virus gene copies/cell; (ii) the number and type of virus genes in each segment of virus nucleic acid; (iii) the location (e.g. integrated or episomal) of the virus genetic information; or (iv) some combination of these factors. Our inability to detect HBcAg in the treated cell cultures may indicate that the cells do not contain a complete virus genome and/or are defective in gene expression. Clearly, these and other human hepatoma cell lines allow further studies of the relation between HBV and hepatocellular carcinoma and of the mechanisms controlling expression of the virus antigens.

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