Effects of Glycosylation Inhibitors on the Expression of Polyalbumin Receptors by Hepatitis B Surface Antigen Produced in vitro

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SUMMARY

In this study we examined the role of secondary modifications in the expression of polyalbumin receptors by hepatitis B surface antigen (HBsAg) produced in vitro. Several clones isolated from 3T3 mouse fibroblasts after transfection with the hepatitis B virus genome produced HBsAg with marked variation in the expression of polyalbumin receptors. Treatment of the cells with glycosylation inhibitors (tunicamycin, glucosamine) resulted in loss of the 27000 mol. wt. HBsAg glycopolypeptide, concomitantly with a marked increase in polyalbumin receptors. These data suggest that glycosylation regulates the activity of polyalbumin receptors associated with native HBsAg.

INTRODUCTION

Hepatitis B surface antigen (HBsAg) from hepatitis B virus (HBV) carriers who are positive for HBeAg has been reported to display higher activity of receptors for polymerized human serum albumin (pHSA-R) than that obtained from carriers positive for antibody to HBeAg (anti-HBe) (Imai et al., 1979; Neurath & Strick, 1979; Hansson & Purcell, 1979). HBsAg from HBeAg-positive carriers was also found to contain significantly more GP31 and GP35 glycopolypeptides than that from anti-HBe-positive carriers (Machieta et al., 1983; Stibbe & Gerlich, 1982). The association of the HBeAg-positive state with large amounts of GP31 and GP35 is consistent with the finding that a 55 amino acid polypeptide of GP31, which is encoded by the pre-S region of the HBV genome, bears the receptor for pHSA (Machida et al., 1984). In marked contrast, Matiu et al. (1980) detected pHSA-R activity with the P22 and P68 peptides purified from HBsAg obtained either from HBeAg-positive carriers or from the medium of cultured PLC/PRF/5 human hepatocarcinoma cells. While the reasons for this discrepancy are not apparent, it should be pointed out that in the above-mentioned studies, denatured polypeptides were examined for pHSA-R activity, making it impossible to assess the significance of the conformational status of the antigens in the expression of pHSA-R in the native state. Therefore, in order to investigate the role of secondary modifications on the expression of pHSA-R by native HBsAg we examined the effects of glycosylation inhibitors on the expression of pHSA-R in vitro.

METHODS

Cell cultures. For this study, we used 3T3 mouse fibroblasts transfected with a cloned HBV genome and containing full copies of the HBV genome (4.10 cells) prepared as previously described (Christman et al., 1982). The 4.10 cells were subcloned by limiting dilution in Falcon 96-well microculture plates. Isolated clones were further cultured to confluence in 25 cm² flasks and the supernatants were assayed for HBsAg and pHSA-R. For the purification of HBsAg, clones selected after the third passage were grown to confluence in 150 cm² flasks containing 30 ml Dulbecco’s minimal essential medium (DMEM) with 10% foetal calf serum (FCS; Gibco) and 40 μg/ml methotrexate. For the purification of HBsAg after glucosamine (Sigma) or tunicamycin (Calbiochem) treatment, 150 cm² confluent flasks were changed to the above medium supplemented with 20 mM-glucosamine or 3 μg/ml tunicamycin and harvested after 2 days. The above concentrations of glucosamine and tunicamycin were
used because they were found to eliminate completely GP27 without significantly affecting cell viability up to 48 h (cell viability >90% by trypan blue exclusion).

**Purification of HBsAg.** For the preparation of anti-HBs affinity columns, goat anti-HBsAg (Dako, Westbury, N.Y., U.S.A.) was linked to cyanogen bromide (CNBr)-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. After coupling, the gel was equilibrated with 0.5 M-NaCl, 0.01 M-Tris-HCl pH 7.4 and stored until use. For the purification of HBsAg, 200 ml of medium collected as described above was precipitated with 80% ammonium sulphate. The precipitate was suspended in 0.1 vol. 0.5 M-NaCl, 0.01 M-Tris-HCl pH 7.4 and extensively dialysed against the same buffer. Ten to 20 ml of this preparation was applied to a small column (3.5 ml bed vol.) and washed consecutively with 150 ml 1 M-NaCl with 0.01 M-Tris-HCl pH 7.4, and 100 ml 1 M-NaI. Finally, the column was eluted with 5 M-NaI, the collected fractions were dialysed extensively against distilled water and then assayed for HBsAg by radioimmunoassay (AusRia II, Abbott Laboratories, North Chicago, Ill., U.S.A.). Positive fractions were pooled, lyophilized and resuspended in 3% SDS, 5% 2-mercaptoethanol.

For the preparation of pHSA in solid phase, pHSA was prepared by cross-linking with glutaraldehyde as previously described (Thung & Gerber, 1981a), and linked to CNBr-activated Sepharose 4B bearing a six-carbon spacer group (CH-Sepharose 4B, Pharmacia).

The amount of HBsAg binding to immobilized pHSA was calculated as follows. First, we determined the amount of pHSA coupled to CH-Sepharose 4B that was capable of removing all HBsAg with detectable pHSA-R activity from a given amount of HBsAg. Then, we incubated a threefold excess of pHSA linked to CH-Sepharose 4B with that amount of HBsAg for 3 h. After removing the Sepharose by centrifugation, we determined the amount of bound HBsAg by subtracting the unbound HBsAg from the total amount mixed with the Sepharose. After washing, the Sepharose-bound HBsAg could be fully recovered by elution with 5 M-NaI.

**SDS-polyacrylamide gel electrophoresis.** Electrophoresis was performed on 1.5 mm-thick 5 to 15% (w/v) SDS-polyacrylamide slab gels (40:1 by weight, acrylamide : bisacrylamide) as previously described (Kasambalides & Lanks, 1979). After electrophoresis, the gels were soaked in 50% methanol, 10% acetic acid overnight and then stained with silver by the method of Oakley et al. (1980). Molecular weight markers were bovine serum albumin (66000), ovalbumin (45000), carbonic anhydrase (29000) and soybean trypsin inhibitor (20100).

**Immunofluorescence.** The cells were grown to confluence in eight-chamber culture slides (Lab-Tek, Miles Laboratories). For detection of pHSA-R, the cells were fixed in acetone at -70 °C and incubated with pHSA (1 mg/ml) followed by fluorescein-conjugated rabbit anti-human albumin. Control slides were incubated with monomeric human albumin. For HBsAg detection, slides were incubated with goat anti-HBs followed by fluorescein-conjugated rabbit anti-goat IgG. A detailed description of these procedures and of the controls has been published elsewhere (Thung & Gerber, 1981b).

**Radioimmunoassays.** Quantifications of HBsAg were performed by AusRia II using the hepatitis B vaccine Heptavax-B (Merck, Sharp & Dohme, West Point, Pa., U.S.A.) containing 20 μg HBsAg per ml as standard (Gilja et al., 1985). The curve obtained showed a linear portion in the range of 5 to 100 ng/ml. Since our standard consisted of the final product bound to alum, some underestimation of the absolute amount of antigen secreted in the medium may have occurred. Equal amounts of HBsAg, relative to the vaccine standard, were assayed for pHSA-R activity by a modification of the procedure of Milich et al. (1981) as previously described (Gilja et al., 1985). Polyalbumin binding activity is expressed as c.p.m. bound in the complete (positive) assay/c.p.m. bound by the mean negative control (the P/N ratio). A value of over 2.1 was considered positive. The P/N ratio for a given amount of HBsAg varied by ±1 to 5% from experiment to experiment. HBeAg was detected by Abbott-HBe radioimmunoassay (Abbott Laboratories).

**RESULTS**

Clones D7, C5 and C6, isolated from 4.10 cells, produced high, intermediate and low amounts of HBsAg respectively, and exhibited marked variation in the expression of pHSA-R activity (Table 1). The effects of tunicamycin and glucosamine, two well-known inhibitors of glycoprotein synthesis (Hubbard & Ivatt, 1981), on the expression of HBsAg-associated pHSA-R produced by clone C5 are shown in Fig. 1. Both drugs induced a marked increase in the expression of pHSA-R when compared to untreated controls at equal amounts of HBsAg. Since glucosamine was found to have a more pronounced effect, it was used for all subsequent experiments. A similar effect of glucosamine on HBsAg-associated pHSA-R was also seen with clones D7 and C6 (Table 1).

The percentage of HBsAg from each clone that expressed pHSA-R activity, and therefore bound to pHSA linked to CH-Sepharose 4B with or without glucosamine treatment, is shown in Table 1. These data correlate well with the results of the radioimmunoassay for pHSA-R and demonstrate that HBsAg-associated pHSA-R having a high P/N ratio exhibited a high
**Polyalbumin receptors of HBsAg**

Polyalbumin receptors of HBsAg derived from clone C5 without treatment (O), after tunicamycin treatment (▲) or after glucosamine treatment (●). Solid-phase pHSA was incubated with various concentrations of HBsAg (abscissa). Polyalbumin binding activity is expressed as P/N (ordinate).

**Fig. 1.** Effect on pHSA binding of concentration of HBsAg derived from clone C5 without treatment (O), after tunicamycin treatment (▲) or after glucosamine treatment (●). Solid-phase pHSA was incubated with various concentrations of HBsAg (abscissa). Polyalbumin binding activity is expressed as P/N (ordinate).

<table>
<thead>
<tr>
<th>Clone</th>
<th>Secreted HBsAg (pg/cell/day)</th>
<th>Treatment</th>
<th>pHSA-R (P/N at 70 ng/ml HBsAg)</th>
<th>% HBsAg binding to pHSA-CH-Sepharose 4B</th>
</tr>
</thead>
<tbody>
<tr>
<td>D7</td>
<td>1.94</td>
<td>None</td>
<td>7</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glucosamine</td>
<td>9.5</td>
<td>76</td>
</tr>
<tr>
<td>C5</td>
<td>1.36</td>
<td>None</td>
<td>5.8</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glucosamine</td>
<td>11.5</td>
<td>57</td>
</tr>
<tr>
<td>C6</td>
<td>0.07</td>
<td>None</td>
<td>2.5</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glucosamine</td>
<td>5.8</td>
<td>46</td>
</tr>
</tbody>
</table>

The percentage binding to pHSA and that glucosamine treatment resulted in increased binding. The percentage of cells of clone C5 producing HBsAg and pHSA-R activity was determined by immunofluorescence. On a scale of intensity ranging from 0 to 3+, about 90% of the cells of clone C5 showed 2+ staining for HBsAg, which increased to 3+ in 100% of the cells after glucosamine treatment (Fig. 2). At the same time, 50% of the cells showed 1+ staining for pHSA-R without treatment, increasing to 3+ in 90% of the cells after glucosamine treatment (Fig. 3). The amount of HBsAg secreted by the cells without treatment or after glucosamine or tunicamycin treatment remained the same as determined by radioimmunoassay (data not shown).

The peptide pattern of HBsAg purified from clone C5 with or without glucosamine treatment was analysed by SDS–PAGE and silver staining (Fig. 4). Without glucosamine treatment both major components, P23 and GP27, were present. Minor bands of 43000 and 46000 mol. wt. and bands in the 46000 to 66000 mol. wt. region were also seen (Fig. 4, lane 1). In our experience, however, the intensity of the various bands after silver staining shows a narrow linear range and hence accurate calculation of the relative abundance, particularly of the minor bands, is difficult. Optimal visualization of the major bands was achieved before the minor bands became apparent. Fig. 4, lane 2, for example, contained an equal amount of the same sample applied to lane 1 but was developed for a shorter time. We could not identify any consistent differences in the presence or intensity of these bands in HBsAg from the various clones, even when we overloaded the gels, which, however, inevitably resulted in distortion of the low mol. wt. viral polypeptides. As expected, glucosamine treatment virtually eliminated GP27 (Fig. 4, lane 3). Although not shown here and difficult to demonstrate, our impression from overloaded gels was that glucosamine treatment resulted in a decrease of the 46000 but not of the 43000 mol. wt.
Fig. 2. HBsAg in the cytoplasm of clone C5 cells. Intensity of staining is significantly increased after glucosamine treatment of cultured cells (a) compared to the control cells (b). Indirect immunofluorescence with goat anti-HBs. Bar markers represent 10 μm.
Fig. 3. Polyalbumin binding activity in the cytoplasm of clone C5 cells. The number of cells that bind polyalbumin is markedly increased after glucosamine treatment (a) compared to the control cells (b). Fluoresceinated anti-human albumin was applied after incubation with polyalbumin. Bar markers represent 10 µm.
band. No qualitative differences were seen in the higher mol. wt. bands. We did not detect any peptides in the 31000 to 34000 mol. wt. region with or without glucosamine treatment. The peptide pattern of clones D7, C5 and C6 was identical with or without glucosamine treatment (data not shown).

DISCUSSION

The results presented in this study indicate that unglycosylated HBsAg exhibits much higher pHSA-R activity than the glycosylated form. These findings suggest that glycosylation, and possibly the conformational state of HBsAg, is the way in which the activity of pHSA-R is regulated in the native state.

Our immunofluorescence studies show that after glucosamine treatment both the intensity of staining and the percentage of cells with pHSA-R activity increase dramatically. This finding suggests that the same cells are capable of producing HBsAg with high or low pHSA-R activity, which is further supported by the increase in the percentage of HBsAg capable of binding to pHSA linked to CH-Sepharose 4B after glucosamine treatment. The increase in the intensity of immunofluorescence staining for HBsAg after glucosamine treatment is difficult to interpret. Since our findings and those of others (Patzer et al., 1984) suggest that inhibition of glycosylation does not affect secretion of HBsAg by mammalian cells, the increased immunofluorescence may not be due to intracellular retention of HBsAg. Although it has been shown that neuraminidase and galactose oxidase treatment do not alter the antigenicity of HBsAg particles (Skelly et al., 1978), it is possible that unglycosylated HBsAg exhibits more antigenic sites before its assembly into particles than the glycosylated form.
Attempts by others to identify the pHSA-R binding region of one of the polypeptides of HBsAg purified after denaturation and SDS-PAGE have resulted in contradictory findings.

Matiu et al. (1980) using non-immunological methods detected pHSA-R in the P22 and P68 polypeptides. In support of this hypothesis, Neurath (1984) noted similar amino acid sequences along the HBsAg P23 polypeptide as compared to human serum albumin. In marked contrast, Machida et al. (1983, 1984) detected pHSA-R activity in a 55 amino acid residue of GP31 which is encoded by the pre-S region of the HBV genome. It is possible that all three polypeptides after denaturation bind to pHSA-R and that Machida et al. did not detect binding on P22 and P68 because the antibody they used in their radioimmunoassay was not raised against the denatured polypeptides. This was suggested by Neurath et al. (1984) who reported that this 55 amino acid polypeptide contains highly immunogenic epitopes and that after denaturation, anti-HBs recognizes only this polypeptide. It has been suggested recently that the open reading frame of the HBV genome encoding HBsAg may be divided into three independently expressed parts: pre-S-1 coding for a large surface protein (P39) and its glycosylated form (GP42), pre-S-2 coding for a middle surface protein containing a one-mannose glycan (GP33) and its further glycosylated form (GP36), and gene S coding for the P23 peptide and its glycosylated form GP27 (Heermann et al., 1984). Further analysis of the carbohydrate content of these peptides showed that all three of them (GP42, GP36 and GP27) have in common a complex glycan at asparagine 146. Furthermore, GP33 and GP36 have a mannose-rich glycan at the asparagine 51 position upstream from the start of P23 (Heermann et al., 1984, Stibbe & Gerlich, 1983). Our results cannot resolve the controversy of whether polyalbumin binds to denatured P23 and P68 as reported by Matiu et al. (1980) or to GP31 as reported by Machida et al. (1984). Although we cannot detect GP31 by silver staining of the gels, it is conceivable that minute amounts are produced by the cloned cells. In addition, it is possible that the minor bands observed in the 43000 to 46000 mol. wt. range represent the large surface protein encoded by the pre-S-1 region which contains also the 55 amino acids of the pre-S-2 region reported to bind pHSA by Machida et al. (1983). Alternatively, they might represent aggregates of P23 and GP27, respectively. The proteins in our gels of mol. wt. higher than 46000 are presently thought to be dimers (Heermann et al., 1984; Koistinen, 1980). It is also possible that artefacts contribute to the increased intensity of these bands after prolonged exposure. Tasheva & Dessev (1983) reported that 2-mercaptoethanol causes the appearance of artefactual, non-protein, bands in the 54000 to 62000 mol. wt. region, particularly when silver stain is used. We confirm this finding, since we have observed intense bands in this region after prolonged exposure, even when we apply only sample buffer and mercaptoethanol in the gel slots. The biological significance of demonstrating binding of pHSA to a viral polypeptide after denaturation and purification from SDS-polyacrylamide gels is not clear. Since it has not been shown that the binding site of HBsAg is a specific receptor for pHSA, it is possible that more than one binding site exists, and that only one or a few are active after denaturation. Our studies demonstrate that tunicamycin and glucosamine treatment result in increased expression of pHSA-R by secreted HBsAg. Tunicamycin is known specifically to inhibit the transfer of N-acetylglucosaminyl-1-phosphate to phosphodolichol, thus blocking the first step in the phosphodolichol pathway (Hemming, 1983). As expected, this results in complete elimination of GP27.

Recent results are compatible with a model in which the NH2- and COOH-terminal parts of the two major peptides of HBsAg are buried within the lipid matrix of HBsAg particles, and residues 122 to 155 occupy an exposed region in the overall morphology of the particle (Cavilanes et al., 1982). This exposed region is the major attachment site for sialic acid-containing carbohydrates and contains an asparagine residue (Asn-146) in the glycosylation sequence (Asp-X-Thr/Ser) (Peterson et al., 1982). If, indeed, P23 does bind pHSA, it is tempting to speculate that variations in the glycosylation of this region modulate the expression of pHSA-R. If, however, the large pre-S-1 and middle pre-S-2 sequences are also present in our clones, then tunicamycin treatment should also inhibit their glycosylation. Both of these peptides are exposed on the outer surface of the viral envelope (Heermann et al., 1984) and lack of carbohydrate might cause profound alterations in their conformational state and orientation in the viral envelope. Feitelson et al. (1983), after detailed two-dimensional tryptic peptide
mapping, suggested the existence of heterogeneity in glycosylation when they compared the major pair of polypeptides from different HBsAg isolates. It is possible that heterogeneity serves as a fine regulatory mechanism for the expression of pHSA-R in the native state. Recently, it has been suggested that HBsAg with high pHSA-R activity might be a particularly effective vaccine (Michel et al., 1984). It has also been shown that carbohydrate dramatically influences immune reactivity to viral glycoprotein antigens (Alexander & Elder, 1984). HBsAg from which carbohydrate has been removed might be particularly effective in eliciting a vigorous immune response against the pHSA receptor.

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REFERENCES


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