Sialyl Residues in Hepatitis B Antigen: their Role in Determining the Life Span of the Antigen in Serum and in Eliciting an Immunological Response

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SUMMARY

Hepatitis B surface antigen was adsorbed to insolubilized sialic acid-specific haemagglutinin isolated from the haemolymph of Limulus polyphemus. Treatment of the antigen with Vibrio cholerae neuraminidase (EC 3.2.1.18) resulted in the release of sialic acid and in an increase of the isoelectric point from pH 4.35 (for subtype ad) or 4.9 (for subtype ay) to pH 5.45. Treated, but not untreated, antigen incorporated $[^{14}C]$-sialic acid when incubated at 37 °C with sialyl transferase (EC 2.4.99.1) and cytidine-5'-monophosphate-$[^{14}C]$-sialic acid. The major portion of $[^{14}C]$-sialic acid was linked to a glycoprotein with an apparent mol. wt. of $26 \times 10^3$. De-sialylated antigen had a drastically reduced in vivo life span in rabbit plasma and elicited a higher humoral antibody response than intact antigen (subtype ad). Antigen-stimulated proliferation of lymphocytes, measured 3 months after immunization, was observed only with cells from rabbits injected with neuraminidase-treated antigen.

INTRODUCTION

Cumulative evidence suggests that sialyl residues of sialoglycoproteins represent discriminatory tags determining the type of interactions between these glycoproteins and other macromolecular structures, and consequently the fate of the glycoproteins in vivo. This conclusion is based on the following independent findings: (a) selective removal of sialyl residues results in accelerated elimination from the circulation of plasma glycoproteins (Morell et al. 1968; Morell et al. 1971; Rogers & Kornfeld, 1971; Regoeczi, Hatton & Wong, 1974) and erythrocytes (Jancik & Schauer, 1974), and in an increased accumulation of lymphocytes in the liver (Woodruff & Gesner, 1969); (b) sialyl residues of the virus glycoprotein play an essential role in initiation of infection with vesicular stomatitis virus (Schloemer & Wagner, 1974) and possibly with other viruses containing sialoglycoproteins; and (c) treatment with neuraminidase increased the immunogenicity of some tumour cells (Weiss, 1973). Since hepatitis B surface antigen (HB$_s$Ag) contains carbohydrates (Bond, 1972; Cawley, 1972; Burrell et al. 1973; Neurath, Prince & Lippin, 1973) linked to three distinct polypeptides (Chairez et al. 1973), we wished to establish whether sialyl residues were present in HB$_s$Ag, and if so, to determine their biological significance.
METHODS

Purification and determination of HBsAg. Spherical HBsAg particles approx. 20 nm in diam. were purified from pooled sera of HBsAg-positive blood donors by precipitation with polyethylene glycol, followed by chromatography on insolubilized concanavalin A, molecular exclusion chromatography, rate zonal and isopycnic sedimentation as described before (Neurath, Prince & Lippin, 1974). HBsAg was determined by radioimmunoassay (RIA; Ausria-125, Abbott Laboratories, North Chicago, Illinois) and subtyped by haemagglutination (HA) inhibition (Prince, Brotman & Ikram, 1972). The amount of HBsAg was expressed in RIA units, defined as the volume in nl of a HBsAg-positive serum, used as a standard, required to obtain the same RIA result as with a test sample. This value was determined from a standard curve relating radioactivity (ct/min) to the dilution of the standard serum in human serum negative for both HBsAg and antibodies to HBsAg (HBsAb). The sensitivity limit of RIA was 8 RIA units, corresponding to approx. 10 ng/ml (Ling & Overby, 1972).

Titration of HBsAb and measurement of HBsAg-induced proliferation of lymphocytes. HBsAb was determined by a RIA-inhibition test in sera of rabbits 10 days after intravenous injection of HBsAg. Serial twofold dilutions of the sera in normal rabbit serum were mixed with 200 RIA units of HBsAg and incubated for 1 h at 37 °C. Then each mixture was tested by RIA. The HBsAb titre was expressed as the serum dilution at which a 50% reduction of ct/min occurred as compared with ct/min for 200 RIA units of HBsAg alone.

In vitro stimulation of DNA synthesis in peripheral lymphocytes was measured as described recently (Pellegrino et al. 1973). Twenty-two cultures were used per animal. Each culture contained 0.1 ml of blood collected from rabbits 3 months after immunization with HBsAg. To 10 cultures each were added 30 μg of purified HBsAg; another 10 cultures served as controls; the remaining 2 cultures received 1 μl of phytohaemagglutinin-P (Difco Laboratories, Detroit, Michigan). All cultures were incubated 4 days at 37 °C. Following addition of 0.5 μCi of [125I]-iododeoxyuridine ([125I]-IUdR; 9.8 μCi/μg; Amersham-Searle, Arlington Heights, Illinois) per culture, incubation at 37 °C was continued for an additional 16 h. The stimulation of DNA synthesis was assessed by calculating the average ratio of [125I]-IUdR incorporated into HBsAg- or phytohaemagglutinin-stimulated and control lymphocytes.

Preparation of insolubilized Limulus polyphemus haemagglutinin. The haemolymph from Limulus polyphemus, obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts, was centrifuged in the Spinco rotor no. 30 for 21 h at 30000 rev/min. The pellet which contained haemocyanin, the most abundant protein of the haemolymph (Marchalonis & Edelman, 1968), was discarded; the supernatant fluid containing the haemagglutinin was concentrated fourfold by ultrafiltration and stored frozen at -70 °C. A 6 ml sample, containing 80 mg of protein, was mixed with 6 ml of 0.1 M-phosphate buffer–0.5 M-NaCl, pH 8.0, clarified by centrifuging at 8000 rev/min for 20 min and mixed with 2 g of CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) at 24 °C for 16 h. The gel was washed with 100 ml of 0.14 M-NaCl–0.01 M-tris, pH 7.2, (TS) and then mixed for 2 h at 24 °C with 1 M-ethanolamine–0.1 M-CaCl2, pH 8.0. Finally, the gel was washed with 200 ml of TS–0.1 M-CaCl2. Thirty mg of protein were linked per g of Sepharose.

Treatment of HBsAg with neuraminidase and preparation of [14C]-sialic acid-labelled HBsAg. In various experiments 2 to 10 mg quantities of HBsAg (subtype ad = HBsAg/ad) in 12 ml of 0.1 M-acetate–1 mM-CaCl2, pH 5.5, were mixed with 500 units of protease-free Vibrio cholerae neuraminidase (Behring Diagnostics, Somerville, New Jersey) and incubated 16 h at 37 °C. Control HBsAg was incubated under the same conditions in the absence of
neuraminidase. HBsAg was pelleted by centrifuging at 65,000 rev/min for 3 h in the Spinco rotor SW65. The supernatant fluid was used for sialic acid determinations (Aminoff, 1961) and the pellets were resuspended in 6 ml of TS. Two and a half ml samples of the neuraminidase-treated HBsAg were layered on top of 2 ml of 2.8 M-glycerol in TS and re-centrifuged under the conditions given above. To label with [14C]-sialic acid, the pellet was resuspended in 2.5 ml of TS, mixed with 0.8 ml of sialyl transferase, prepared from rabbit liver as described by Hickman et al. (1970), with 0.4 μCi of cytidine-5'-monophosphate-4,5,6,7,8,9-[14C]-sialic acid (CMP-[14C]-sialic acid, 217 mCi/mmol; New England Nuclear, Boston, Massachusetts) and incubated for 1 h at 37 °C. Subsequently, the mixture was layered on top of 0.5 ml of 2.8 M-glycerol in TS and centrifuged in the rotor SW65 for 40 min at 40,000 rev/min. Labelled HBsAg from the supernatant fluid was pelleted by centrifuging at 65,000 rev/min for 4 h, resuspended in TS and submitted to 2 successive rate zonal sedimentations under conditions given for Fig. 3. In addition to neuraminidase-treated HBsAg, intact HBsAg and sialyl transferase alone were also incubated with CMP-[14C]-sialic acid and further processed as described.

Isoelectric focusing. The LKB 8101 column (LKB Produkter AB, Bromma, Sweden) was used for isoelectric focusing of untreated and neuraminidase-treated HBsAg/ad (200 μg) and HBsAg/lay (10 μg). A 1% (v/v) carrier ampholyte covering the pH range of 3 to 10 in a 0 to 6.8 M-glycerol gradient was used. Two ml samples in 3.4 M-glycerol were applied in the middle of the gradient. Electrofocusing was performed at 300 V for 72 h at 12 °C. Samples of 2 ml each were collected. Their pH was measured with the Radiometer type TTTIC titrator (Radiometer, Copenhagen, Denmark). Samples were diluted 10 to 50-fold in normal serum for RIA assays of HBsAg.

Polyacrylamide gel electrophoresis. Labelled HBsAg or crude sialyl transferase was pelleted by centrifuging at 65,000 rev/min for appropriate lengths of time and dissolved in 50 to 200 μl of 0.1 M-sodium phosphate-5 M-urea-0.087 M-SDS-0.13 M-mercaptoethanol, pH 7.2, and boiled for 3 min. The samples were electrophoresed in 8% flat polyacrylamide gels under conditions described by Neurath et al. (1972), except that the gels contained 5 M-urea. The measurement of radioactivity along the gel and the assignment of mol. wt. to species with distinct electrophoretic mobilities were performed as before (Neurath et al. 1972).

Electron microscopy. The loop drop method (Howatson, 1969) was used to count HBsAg particles.

RESULTS

Evidence for sialyl residues in HBsAg

Affinity chromatography of HBsAg on an adsorbent specific for sialyl residues combined with a RIA for HBsAg was expected to answer the question whether or not such residues were exposed on the surface of HBsAg without having to resort to chemical analyses requiring large quantities of HBsAg. HBsAg/ad was adsorbed on insolubilized Limulus polyphemus agglutinin specific for sialyl residues (Sharon & Lis, 1972), and was eluted with a buffer containing sialic acid either under conditions given for Fig. 1 or more slowly at 4 °C with TS-0.1 M-CaCl2-0.035 M-sialic acid. The fraction of HBsAg which did not attach to the column was re-chromatographed. The major portion of HBsAg was again adsorbed to the column, indicating that the antigen preparation did not consist of two kinds of particles, one with exposed sialyl residues, and the other devoid of such residues. These results may be better explained by the establishment at 37 °C of an equilibrium between adsorbed and unadsorbed HBsAg.
Fig. 1. Affinity chromatography of HBsAg/ad on Sepharose 4B to which Limulus polyphemus haemagglutinin was linked. Fifty thousand RIA units of HBsAg/ad were applied at 37 °C to a 1 g column pre-washed with 5 ml of 0.145 mM-human serum albumin in TS-0.1 M-CaCl₂. The column was kept 1 h each at 37 °C and subsequently at 4 °C and washed with TS-0.1 M-CaCl₂ at 4 °C. HBsAg/ad was eluted at 37 °C with TS-2 M-CaCl₂-0.035 M-sialic acid applied at the point indicated by the arrow. One ml fractions were collected.

Spectrophotometric analyses of supernatant fluids after pelleting neuraminidase-treated HBsAg revealed that approx. 1.9 μg of sialic acid were released from 100 μg of HBsAg/ad. Treatment with neuraminidase resulted in an increase of the isoelectric point of HBsAg from pH 4.35 (for subtype ad) or 4.9 (for subtype ay) to pH 5.45 (Fig. 2). The former two values agree with published data for the major portion of HBsAg/ad or ay (Howard & Zuckerman, 1973).

Enzymic transfer of [¹⁴C]-sialic acid to HBsAg

Identical amounts of untreated and neuraminidase-treated HBsAg/ad were incubated with sialyl transferase and CMP-[¹⁴C]-sialic acid, subsequently purified as described in Methods and centrifuged in a glycerol gradient. Only with treated HBsAg/ad was radioactivity recovered in the gradient fractions containing the antigen, suggesting that sialic acid was transferred to de-sialylated but not to intact HBsAg/ad (Fig. 3). Comparison of the radioactivity and the number of HBsAg particles in pooled fractions 8 to 10 revealed that an average of 100 sialic acid molecules were incorporated into a single HBsAg/ad particle.

To show that [¹⁴C]-sialic acid became covalently linked to HBsAg/ad glycoproteins and to identify the sialoglycoprotein(s), labelled HBsAg/ad was dissociated in 5 M-urea-0.13 M-mercaptoethanol-0.087 M-SDS and electrophoresed in an 8% polyacrylamide gel. The major part of the radioactivity was recovered in a component with an electrophoretic mobility expected for a polypeptide having a mol. wt. of 26 × 10³ (Fig. 4), identical with the size of the smaller of the two major (glyco)proteins of HBsAg (Gerin, Holland & Purcell, 1971; Chairez et al. 1973). The remainder of the radioactivity was detected in regions of the gel in which polypeptides with mol. wt. of 31 to 35 and 45 × 10³, respectively, would be recovered. The absolute mol. wt. of the sialoglycoproteins may differ from the values based on calibrating the gel with protein standards because of possible anomalies in the electrophoretic mobility of SDS-glycoprotein complexes in relation to protein-SDS complexes.
Fig. 2. Isoelectric focusing of untreated and neuraminidase-treated HB,Ag. 
O--O, untreated HB,Ag; ●--●, neuraminidase treated HB,Ag.
Fig. 3. Distribution of [14C]-sialic acid label and of HBsAg/ad in fractions after rate zonal sedimentation for 2 h at 56000 rev/min in the Spinco rotor SW65. HBsAg/ad treated with neuraminidase and subsequently with sialyl transferase and purified as described in Methods was centrifuged in a 1.4 M to 6.8 M-glycerol gradient in TS. The results of the second of two consecutive identical sedimentations are presented. After the first sedimentation, 50% of the radioactivity was recovered in the bottom third of the gradient in which HBsAg was not detected. This radioactive material corresponded to crude sialyl transferase (see Fig. 4). O—O, [14C]-sialic acid; O—O, % HBsAg recovered in each fraction.

This reservation would also apply to results of previous studies (Gerin et al. 1971; Chairez et al. 1973). The distribution along the gel of sialoglycoproteins, labelled as the result of endogenous transfer of [14C]-sialic acid into components of the sialyl transferase preparation differed markedly from the pattern observed for HBsAg/ad. This excluded the possibility that any of the labelled components attributed above to HBsAg were contaminants originating from the crude enzyme preparation.

Similar experiments were carried out with HBsAg/ay. Difficulties were encountered in separating the neuraminidase-treated HBsAg/ay from components of the sialyl transferase. Labelled glycoproteins from both HBsAg/ay and the enzyme preparation appeared in polyacrylamide gels after electrophoresis of the incompletely purified [14C]-sialic acid-labelled HBsAg/ay. However, it was concluded that: (a) the major labelled component of HBsAg/ay had the same electrophoretic mobility as the major labelled sialoglycoprotein of HBsAg/ad and (b) approx. 20 sialic acid molecules were incorporated into a single HBsAg/ay particle. The smaller number of sialic acid molecules per antigen particle transferred into HBsAg/ay as compared to HBsAg/ad appears to agree with the comparatively higher isoelectric point of HBsAg/ay. Treatment with neuraminidase did not convert HBsAg/ad into HBsAg/ay.
Sialyl residues in hepatitis B antigen

Crude sialyl transferase

Fig. 4. Polyacrylamide gel electrophoresis of purified [¹⁴C]-sialic acid-labelled HB₄Ag/ad and of crude sialyl transferase recovered from the bottom third of the gradient after rate zonal sedimentation under conditions given for Fig. 3. Quantities corresponding to 5000 ct/min each were electrophoresed.

Altered biological properties of neuraminidase-treated HB₄Ag

De-sialylation of various glycoproteins enhances their removal from the circulation and their uptake in the liver (Morell et al. 1968, 1971; Rogers & Kornfeld, 1971; Regoezzi et al. 1974). Results summarized in Fig. 5 show that treatment with neuraminidase shortened the in vivo life span of HB₄Ag/ad in rabbit serum approx. 10- to 20-fold. Similar results were obtained with HB₄Ag/ay.
Fig. 5. Clearance from serum of untreated and neuraminidase-treated HBsAg/ad. Each point represents the average value for 6 rabbits. The length of the vertical bars corresponds to \( \pm 2 \) standard deviation. Injections (0.1 ml = 100 \( \mu \)g of HBsAg) were made in the ear veins of New Zealand white rabbits weighing about 2 kg. Ten times smaller doses of HBsAg/ad were used in similar experiments. ---, untreated HBsAg; ---, neuraminidase treated HBsAg.

Since the immunogenicity of antigens may depend on the speed of their metabolism (Sela, Mozes & Shearer, 1972), the possibility that sialyl residues may have a role in eliciting an immunological response to HBsAg was investigated. Results in Tables 1 and 2 show that: (a) desialylated HBsAg/ad induced a higher humoral antibody response than untreated HBsAg/ad, and (b) the proliferation of lymphocytes from the majority of test rabbits immunized with neuraminidase-treated HBsAg/ad was stimulated with intact HBsAg/ad while lymphocytes from animals injected with untreated HBsAg/ad were not stimulated. Lymphocytes from both groups of rabbits did not differ significantly in their proliferative response to phytohaemagglutinin.

**DISCUSSION**

The results described here show that sialyl residues are present on the surface of HBsAg and that their removal alters the *in vivo* response of rabbits to HBsAg. The question arises whether or not this finding may be relevant for designs concerning active immunization of humans to hepatitis B and perhaps for immunotherapy of the HBsAg carrier state. Although this question cannot be answered unless further detailed results concerning the cellular and...
Table 1. Difference in humoral immunological response elicited by untreated and de-sialylated HBsAg/ad*

<table>
<thead>
<tr>
<th>HBsAg/ad used for immunization</th>
<th>Untreated</th>
<th>Neuraminidase-treated</th>
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<tr>
<td>HBsAb titre in serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range by RIA inhibition</td>
<td>1:7 to 1:17</td>
<td>1:66 to 1:420</td>
</tr>
<tr>
<td>Mean RIA inhibition titre</td>
<td>(13±1)† (16)‡</td>
<td>(256±15)† (15)‡</td>
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* Rabbits were immunized with HBsAg/ad intravenously as described in text to Fig. 5.
† Standard error of the mean.
‡ Number of rabbits.

Table 2. Difference in immunological response elicited by untreated and de-sialylated HBsAg/ad revealed by HBsAg-stimulated proliferation of lymphocytes*

<table>
<thead>
<tr>
<th>HBsAg/ad used for immunization</th>
<th>Untreated</th>
<th>Neuraminidase-treated</th>
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<tbody>
<tr>
<td>Rₐₜ Pₜ Rₕₜ Pₚ</td>
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<tr>
<td>0.75 ~0.05 1.47 &lt;0.05 2.13 &lt;0.001 5.53 &lt;0.001</td>
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<tr>
<td>0.75 ~0.05 9.20 &lt;0.001 2.00 &lt;0.001 4.66 &lt;0.001</td>
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<td>0.76 ~0.05 5.90 &lt;0.001 1.53 &lt;0.001 2.41 &lt;0.001</td>
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<tr>
<td>0.80 ~0.80 2.66 &lt;0.001 1.50 &lt;0.005 4.24 &lt;0.001</td>
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<tr>
<td>0.95 ~0.50 3.30 &lt;0.001 1.40 &lt;0.01 11.10 &lt;0.001</td>
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<tr>
<td>1.00 &gt;0.90 1.99 &lt;0.001 1.37 ~0.02 8.20 &lt;0.001</td>
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<tr>
<td>1.00 &gt;0.90 4.15 &lt;0.001 1.36 &lt;0.10 6.80 &lt;0.001</td>
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<tr>
<td>1.02 &gt;0.90 10.30 &lt;0.001 1.00 &gt;0.90 1.63 &lt;0.005</td>
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<tr>
<td>1.02 &gt;0.90 5.10 &lt;0.001 0.95 ~0.50 1.83 &lt;0.001</td>
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<tr>
<td>1.06 ~0.80 5.46 &lt;0.001 0.91 ~0.45 2.06 &lt;0.001</td>
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<tr>
<td>0.91¶ ~0.50¶ 2.80¶ &lt;0.001¶</td>
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* Rabbits were immunized with HBsAg/ad intravenously as described in text to Fig. 5.
† Mean ratio of cts/min incorporated into cultures stimulated with HBsAg to cts/min of control cultures, calculated for each of the 21 rabbits.
‡ Probability that Rₐₜ = 1, determined from the 't' test of significance between two means for 18 degrees of freedom.
§ Mean ratio of cts/min incorporated into cultures stimulated with phytohaemagglutinin to cts/min of control cultures.
‖ Probability that Rₕₜ = 1, determined for 10 degrees of freedom.
¶ Values calculated for an unimmunized rabbit.

humoral immunological responses to intact and de-sialylated HBsAg become available, the following comments appear pertinent: (1) The reduction of the life span in plasma of sialoglycoproteins caused by de-sialylation appears to be a general phenomenon, although the magnitude of the reduction may depend on the species-specificity of the glycoprotein (Regoezzi et al. 1974). Therefore, the life span of HBsAg in human plasma should also become reduced after de-sialylation. Consequently, the immune response in humans to de-sialylated HBsAg should not be the same as the response to intact HBsAg. (2) The cell-mediated immune response appears essential for recovery from hepatitis B and for the final clearance of hepatitis B virus and of HBsAg (Dudley, Fox & Sherlock, 1972; Kohler et al. 1974). Since a regression of established tumours was observed after injecting tumour-bearing animals with neuraminidase-treated tumour cells (Simmons & Rios, 1973; Weiss, 1973), it cannot be excluded that de-sialylated HBsAg may elicit a cellular immune response in HBsAg carriers and thus lead, under favourable circumstances, to termination of the proliferation of hepatitis B virus and of HBsAg synthesis.
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REFERENCES


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