Analysis of Hepatitis B Surface Antigen Components
Solubilized with Triton X-100

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SUMMARY

Three glycoproteins of intact hepatitis B surface antigen (HBsAg) with mol. wt. of 32000, 30000 and 28000 respectively were identified by reaction with 125I-concanavalin A (Con A) after separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The antigen was effectively disrupted with Triton X-100 to produce a structure with a sedimentation coefficient of 3.9S. Affinity chromatography of disrupted HBsAg using concanavalin A-Sepharose 4B (Con A-Sepharose) resulted in two fractions. The first contained material which did not bind to the lectin and consisted of a single polypeptide of mol. wt. 64000. Further studies revealed this component to be serologically identical to serum albumin and to lack any affinity for antibody to HBsAg. A comparison of the tryptic peptide map of this polypeptide with that of purified serum albumin demonstrated identical amino-acid sequences. The second fraction contained material which bound to Con A and contained two polypeptides with mol. wt. of 28000 and 23000 respectively. HBsAg reactivity was associated with this fraction. This procedure allows the preparation of HBsAg sub-units in milligram quantities for further immunological studies.

INTRODUCTION

Hepatitis B is presently a major public health problem with an estimated 114 million persistently infected individuals throughout the world (WHO, 1977). Acute or chronic hepatitis B virus infection is manifested by the expression of several gene products, the surface antigen (HBsAg) being particularly abundant as 20 to 25 nm spherical particles. Active virus replication additionally gives rise to detectable numbers of double-shelled particles containing an antigenically distinct core component (HBcAg) within which has been detected a circular DNA molecule with a mol. wt. of $1.8 \times 10^6$ (Robinson, 1977). The limited coding potential of the presumptive genome has generated considerable interest as to the origin of many of the polypeptide species found in the HBsAg-reactive 20 to 25 nm particles. Although first reports described the presence of two major polypeptide species of mol. wt. 24000 to 26000 and 28000 to 30000, additional major components are frequently seen in the mol. wt. range of 35000 to 125000. Many studies have shown that considerable variation exists between analyses obtained with purified HBsAg from different sources and of different serological subtypes (reviewed by Howard & Burrell, 1976); the exact number, size and origin of these polypeptides has yet to be clearly defined. This information is necessary in order to establish whether all virus-specified polypeptides can be accounted for by the limited coding potential of the presumptive genome, estimated at 125000 (Robinson, 1977).
The possible inclusion of host-coded polypeptides in purified preparations of HBsAg particles is an important consideration in view of the potential use of such preparations for the immunoprophylaxis of hepatitis B. That several components are of host origin has been suggested by the finding that purified HBsAg 20 to 25 nm particles may react with a variety of hyperimmune antisera to human protein species (Neurath et al. 1974; Burrell, 1975). Hitherto, attempts to assign virus- or host-specific antigenic determinants to individual polypeptides have been preceded by the recovery of separated components following SDS-PAGE (Dreesman et al. 1975; Shih & Gerin, 1977). In the present study, the non-ionic detergent Triton X-100 was used to solubilize purified HBsAg 20 to 25 nm particles and the resulting components were subjected to chemical and serological analysis.

**METHODS**

**Hepatitis B surface antigen.** The purification of HBs 20 to 25 nm particles from plasma obtained from a persistently infected chimpanzee has been previously described (Skelly et al. 1978). Significant levels of hepatitis B core antigen-associated DNA polymerase activity were present in the original serum together with hepatitis B e antigen as detected by a solid-phase radioimmunoassay system. The final preparation consisted entirely of 20 to 25 nm particles as demonstrated by electron microscopy.

**Radio-iodination of HBsAg.** Purified HBsAg was labelled with radio-iodinated 3-(4-hydroxyphenyl) propionic acid N-hydroxysuccinimide ester (Radiochemical Centre, Amersham, Bucks, U.K.) essentially as described by Bolton & Hunter (1973). Twenty-five μg of protein in 100 μl of 0.2 M-sodium borate buffer, pH 8.5, was added to 250 μCi of dried reagent and incubated overnight at 4 °C. Glycine (500 μl of a 0.2 M solution in borate buffer) was added to react with unconjugated ester and the mixture immediately passed through a commercially prepared column of Sephadex G-25 (Pharmacia Fine Chemicals) equilibrated with 0.05 M-tris-HCl, pH 7.3, containing 0.25% gelatine. The labelled antigen was recovered in the void vol.

**Disruption of HBsAg with Triton X-100.** Labelled or unlabelled HBsAg in 0.01 M-tris-HCl pH 7.3, was disrupted by overnight incubation at 37 °C in the presence of Triton X-100 and NaCl at final concentrations of 2% and 0.5 M respectively.

**Determination of the sedimentation coefficient of disrupted antigen.** Disrupted radiolabelled HBsAg was layered on to a 5 to 20% linear gradient of sucrose buffered with 0.1 M-tris-HCl, pH 7.4, 0.15 M-NaCl, 1 mM-EDTA containing 0.1% Triton X-100 and centrifuged at 229000 g for 16 h in a Beckman SW40 rotor. Fractions (0.5 ml) were collected from the top of the tube and 25 μl samples were counted in a LKB model 1280 gamma counter. The sedimentation coefficient of the antigen was determined using the formula of Martin & Ames (1961) by comparison with the migration in parallel identical gradients of the following standards: immunoglobulin G [mol. wt. 154000, 6.6S (Simons et al. 1973)], Con A [mol. wt. 55000, 4.2S (J. Skelly, unpublished data)] and lysozyme [mol. wt. 14000, 2.1S (Martin & Ames, 1961)].

**Fractionation of disrupted HBsAg on Con A-Sepharose.** A 1 × 10 cm column of Con A-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) was equilibrated with 0.01 M-tris-HCl, pH 7.3 buffer containing 2% Triton X-100, 0.5 M-NaCl, 1 mM-CaCl₂ and 1 mM-MnCl₂. Disrupted radio-iodinated HBsAg was applied to the column which was then washed with buffer to remove unbound material. Bound material was eluted with 0.01 M-tris-HCl, pH 7.3, containing 2% Triton X-100, 0.5 M-NaCl and 5% α-methyl-D-mannoside (α-MM). Fractions (0.75 ml) were collected and suitable samples of each counted for the presence of radioactivity. For fractionation of larger quantities of unlabelled HBsAg, a 27 × 1.5 cm column of Con A-Sepharose 4B was used.

**Removal of Triton X-100 from Con A-Sepharose fractions.** Pooled peak fractions were
first dialysed extensively against 0.01 M-tris-HCl, pH 7.3, at 4 °C; remaining amounts of Triton X-100 were then removed by a modification of the method of Holloway (1973). Washed ‘Biobeads’ SM-2 (Bio-Rad Laboratories, Richmond, California) were pre-incubated for 3 h at room temperature with 5% gelatine and washed in a sintered glass funnel. Moist beads were added to the Triton X-100-containing solution at a concentration of 0.5 g/ml and the mixture incubated on a shaker for 45 min at room temperature. The solution was then separated from the beads. This procedure lowered the Triton X-100 concentration to 0.02%, without any significant binding of labelled protein to the beads.

**Polyacrylamide gel electrophoresis.** The method used for discontinuous-buffer SDS-PAGE, and the conditions for preparation of the samples have been previously described (Skelly *et al.* 1978). Lectin-binding components were detected by labelling with 125I-concanavalin A essentially as described by Neurath *et al.* (1978). Gels were first fixed, stained with Coomassie brilliant blue and destained as previously described (Skelly *et al.* 1978). After several washes over a 48 h period with 0.05 M-tris-HCl, pH 7.5, 0.15 M-NaCl buffer, the gels were sliced into 1 mm fractions, each of which was incubated for 24 h at 4 °C in 100 μl of buffer containing 1 mM-MnCl₂, 1 mM-CaCl₂, 0.5% human haemoglobin and 0.14 μCi/ml of 125I-concanavalin A, labelled by the procedure described for HBsAg. Control gels were treated in a similar fashion, except that MgCl₂ and CaCl₂ were replaced by 5% α-MM. The gel slices were then washed several times in 0.05 M-tris-HCl, 0.15 M-NaCl buffer, drained and counted.

**Radioimmunoprecipitation test.** Samples (25 μl) of the fractionated radiolabelled antigen from columns of immobilized Con A were mixed with 75 μl of 0.05 M-tris-HCl, pH 7.5, containing 1% non-immune rabbit serum (RIP buffer). The following rabbit antisera, diluted 1:100 in RIP buffer, were added in 100 μl samples to duplicate samples: anti-human serum albumin, anti-whole human serum (both from the Miles Laboratories, Slough, England) and anti-HBsAg (Hoechst Pharmaceuticals, Hounslow, Middlesex). After incubation at 4 °C for 18 h, a 20 μl sample of goat anti-rabbit IgG (Hoechst Pharmaceuticals) was added and incubation continued after a further 18 h. The percentage of 125I-radiolabel immunoprecipitated was determined by sequentially counting the supernatants and pellets separated by light centrifugation.

**Purification of chimpanzee serum albumin.** Albumin was purified from the serum of a chimpanzee free from HBV infection by affinity chromatography on Blue Sepharose CL-6B (Pharmacia Fine Chemicals) by the method of Travis & Pannell (1973). A 1 x 10 cm column of Blue Sepharose was equilibrated with starting buffer (0.05 M-tris-HCl, pH 7.5, 0.1 M-KCl). One ml of serum was dialysed against starting buffer for 24 h and applied to the column. The column was washed with starting buffer to remove unbound proteins. The buffer was changed to 0.05 M-tris-HCl, pH 7.5, 1.5 M-KCl to elute albumin. Fractions (1.5 ml) were collected and protein concentration was determined by A₂₈₀. The protein eluted from the column with 1.5 M-KCl gave a single band with a mol. wt. of 64000 in SDS-PAGE. It was radio-iodinated with the Bolton and Hunter reagent by the same procedure as that used for intact HBsAg. The radiolabelled product also gave a single peak in SDS-PAGE and was therefore considered to be free of significant contamination by other serum proteins. In addition, a complete line of identity was observed in agar gel diffusion with human serum albumin and its antibody.

**Tryptic peptide mapping.** The method of Sturman & Holmes (1977) was used. 125I-labelled proteins were exchanged into 1% ammonium bicarbonate on small pre-packed columns of Sephadex G-25. The samples were lyophilized and re-dissolved in 100 μl of 1% ammonium bicarbonate. TPCK-trypsin (Worthington Biochemicals, New Jersey) was added to a final concentration of 50 μg/ml, and the samples were incubated at 37 °C for 24 h. After lyophilization, the samples were re-dissolved in 20 μl of 10% acetic acid and
Fig. 1. SDS-PAGE of purified HBsAg 20 to 25 nm forms. About 25 μg was disrupted and electrophoresed through 10% gels at a constant current of 2.5 mA. Mol. wt. were derived by comparison with the migration of reference proteins of known mol. wt. in a parallel gel. The major polypeptides are indicated by the prefix 'p' and their respective mol. wt. × 10⁻³.

Fig. 2. Labelling of the glycoproteins of HBsAg with ¹²⁵I-con A. A gel similar to that shown in Fig. 2 was sliced and labelled as described in the Methods. The positions of the major polypeptides detected by Coomassie brilliant blue staining are indicated.

spotted on to 20 x 20 cm cellulose plates (Merck, Darmstadt, West Germany). Two dimensional chromatography was carried out according to Bellisario et al. (1973). The first solvent was n-butanol-acetic acid-water (200:30:75, v/v) and the second solvent was n-butanol-pyridine-acetic acid-water (15:10:3:12, v/v). Labelled peptides were detected by autoradiography with Kodak XRP5 film.

RESULTS

SDS-PAGE analysis of intact HBsAg

Purified HBsAg contained four major polypeptides; p64, p30, p28, p23 and additional minor components (Fig. 1). Since the intact antigen binds to Con A-Sepharose (Neurath et al. 1973), the ability of individual separated polypeptides to bind ¹²⁵I-Con A was tested by reacting polyacrylamide gel slices with the labelled lectin. The pattern obtained is shown in Fig. 2. Two major components, p64 and p23, did not bind the lectin. Three polypeptides, p28, p30 and the minor component p32 were labelled. Binding of ¹²⁵I-Con A to all these species was inhibited by 5% α-MM, indicating that the reaction was dependent upon the presence of specific carbohydrate residues in the polypeptides. This experiment demonstrated that HBsAg contains two glycoproteins in addition to p28, previously identified as a glycoprotein by galactose oxidase-tritiated sodium borohydride labelling (Skelly et al. 1978).

Disruption of HBsAg with Triton X-100

Preliminary experiments to compare Triton X-100 and sodium deoxycholate as solubilizing agents for HBsAg indicated that Triton X-100 was the more effective in completely disrupting the 20 to 25 nm form of HBsAg. In order to determine the approximate size of the product, ¹²⁵I-HBsAg was disrupted with 2% Triton X-100, 0.5 M NaCl and centrifuged on 5 to 20% sucrose gradients containing 0.1% Triton X-100 (Simons et al. 1973).
HBsAg components

The sedimentation coefficient of the disrupted material was calculated to be 3.9S (Fig. 3) by comparison with the migration rates of standard proteins run in parallel gradients. In contrast, intact HBsAg was pelleted in these conditions. Treatment with 2% Triton X-100 therefore resulted in the complete disruption of HBsAg into small subunits.

125I-HBsAg, disrupted with 2% Triton X-100 was fractionated on a column of Con A-Sepharose in the presence of the same concentration of the detergent (Fig. 4). About 60% of the radiolabel did not bind to the column; the remaining 40% was eluted with buffer containing 5% α-MM. More than 95% of the radioactivity was recovered, indicating that non-specific binding of material to the gel did not occur.

The polypeptide composition of both fractions obtained by chromatography on Con A-Sepharose were examined by SDS-PAGE. The unbound fraction consisted of p64 only (Fig. 5a). The α-MM eluate consisted predominantly of p28 and p23 (Fig. 5b).

Serological analysis

Purified preparations of HBsAg contain antigenic determinants which react with antisera to several normal human serum components (Burrell, 1975). It was of interest to determine which serological activities were associated with each of the fractions prepared by Con A-Sepharose chromatography. After removal of Triton X-100, samples of each fraction were reacted in a radioimmunoprecipitation test with excess rabbit antibody to HBsAg, whole human serum, or human albumin. The results obtained are shown in Table 1. The unbound fraction was precipitated to a significant extent by anti-whole human serum and by anti-human albumin, but not by anti-HBsAg. In contrast the α-MM-eluted radiolabel was precipitated by anti-HBsAg, but not by anti-whole human serum or anti-albumin. Neither fraction was precipitated by rabbit antisera to a range of other human serum proteins (data not shown). This experiment also demonstrated that disruption with Triton X-100 did not lead to irreversible denaturation of HBsAg antigenic determinants.
Fig. 5. SDS-PAGE of fractions derived from affinity chromatography of disrupted, unlabelled HBsAg on Con A-Sepharose. Four mg of HBsAg was disrupted and fractionated as described in the Methods. The peak fractions from the Con A-Sepharose column were pooled and the Triton X-100 was removed as described in the Methods. The conditions for disruption and electrophoresis were as described in the legend of Fig. 2; (a) unbound fraction; (b) bound fraction.

Table 1. Serological properties of fractions obtained by affinity chromatography of disrupted radiolabelled HBsAg on immobilized Con A

<table>
<thead>
<tr>
<th>Con A-Sepharose fraction</th>
<th>Rabbit serum</th>
<th>Unbound</th>
<th>α-MM eluate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-immune</td>
<td>14·4</td>
<td>4·2</td>
<td></td>
</tr>
<tr>
<td>Anti-whole human serum</td>
<td>85·4</td>
<td>10·5</td>
<td></td>
</tr>
<tr>
<td>Anti-human albumin</td>
<td>84·8</td>
<td>10·6</td>
<td></td>
</tr>
<tr>
<td>Anti-HBsAg</td>
<td>23·2</td>
<td>65·5</td>
<td></td>
</tr>
</tbody>
</table>

* Percentage of radiolabel precipitated (see Methods section).

Tryptic peptide mapping of p64 and purified chimpanzee serum albumin

The previous experiments demonstrated that the portion of disrupted antigen which did not bind to Con A-Sepharose consisted of a single polypeptide with the mol. wt. (64,000) and the antigenic properties of serum albumin. Authentic chimpanzee albumin was prepared
Fig. 6. Tryptic peptide maps of chimpanzee serum albumin (CSA), and p64 obtained from disrupted HBsAg. The conditions for digestion and two-dimensional chromatography are described in the Methods.
from the serum of an uninfected animal as described in the Methods, and iodinated with the Bolton and Hunter reagent. The tryptic digests of the authentic chimpanzee serum albumin and of p64 isolated from HBsAg by disruption and Con A-Sepharose chromatography were compared. Fig. 6 shows that the two proteins had common tryptic peptides and therefore contained identical amino acid sequences.

**Affinity chromatography of HBsAg on Blue Sepharose CL-6B**

It was of interest to determine whether the serum albumin detected in purified preparations of HBsAg was present as a contaminant or was a constituent of the particle itself. HBsAg was therefore chromatographed on Blue Sepharose CL-6B under conditions in which albumin is specifically bound (Travis & Pannell, 1973). Fig. 7 shows that HBsAg was not separated from albumin by this procedure. The antigen was bound to the column in conditions of low salt and subsequently recovered by increasing the salt concentration to 1.5 M. Exactly the same result was obtained with free serum albumin. This result suggested that albumin was an integral component of the HBsAg 20 to 25 nm particle.

**DISCUSSION**

Widely differing results have been reported as to the number and size of the polypeptides found in purified 20 to 25 nm forms of HBsAg (Howard & Burrell, 1976). Early studies on sera from persistently infected human carriers suggested the presence of two or three major polypeptides in the mol. wt. range of 22000 to 40000. Further studies, however, have indicated the presence of as many as six additional polypeptides several of which are present as major components (Chairez *et al.* 1973; Howard & Zuckerman, 1974; MacKay & Burrell, 1976). The unambiguous identification of virus polypeptides has been further complicated by serological studies which indicate the associations of host antigens with extensively purified HBsAg preparations.

Millman *et al.* (1971) found that Tween 80 treatment released material which produced
precipitin lines with antisera to several normal human serum proteins. Neurath et al. (1974) reported that HBsAg particles were selectively retained on immunoadsorbent columns containing immobilized antibodies to the serum proteins albumin, pre-albumin, apolipoproteins C and D and the \( \alpha \)-chain of immunoglobulin G. Burrell (1975) found that purified HBsAg reacted in a radioimmunoprecipitation test with antisera to a number of normal serum components, but he concluded that these reactions were either due to the presence of tightly-bound trace amounts of partly denatured serum components or to a weak cross-reaction with antigen present in normal serum. Hitherto, none of these activities have been correlated with a specific polypeptide. Several laboratories have attempted to define the nature of HBsAg antigenic determinants by assessing the immunogenicity of individual polypeptides recovered from acrylamide gels after solubilization with SDS. Dreesman et al. (1975) examined the serological response in guinea pigs immunized with individual polypeptides separated by preparative gel electrophoresis. Two of five polypeptides derived from the adw subtype with mol. wt. of 35,000 and 40,000 elicited group-reactive (anti-a) antibody. An additional 24,000 mol. wt. polypeptide prepared from HBsAg of subtype ayw was also found to elicit an antibody response. In similar work, Gerin (1974) reported that seven polypeptides ranging in mol. wt. from 23,000 to 95,000 all produced group-specific (anti-a) antibody in guinea pigs. Although these results suggest that each individual polypeptide may contain amino-acid sequences essential for immunoreactivity, results obtained after isolation of components with SDS are difficult to interpret. Renaturation of polypeptides into a native conformation may be inaccurate or incomplete and since antigenicity in globular proteins is dependent almost exclusively on three-dimensional conformation (Arnon & Geiger, 1977) the antigenic determinants of such molecules may differ considerably from those of the original state. The somewhat low affinity of antibodies for components isolated in this way appears to hinder accurate serological analysis (Shih et al. 1978).

This paper reports in detail an alternative approach to the fractionation of HBsAg whereby the serological activity of the antigen is preserved. The purified antigen preparation used in this study has been described previously as containing four major polypeptide species with mol. wt. of 64,000 (p64), 30,000 (p30), 28,000 (p28) and 23,000 (p23) as resolved by SDS-PAGE (Skelly et al. 1978).

The presence of a type A carbohydrate moiety (Johnson & Clamp, 1971) in p28 was unambiguously demonstrated by specific radiolabelling in a reaction using the enzyme galactose oxidase. In the present study, experiments using \(^{125}\)I-concanavalin A in order to identify glycoproteins separated by SDS-PAGE confirmed that this polypeptide was glycosylated (Fig. 3). Other glycoproteins were additionally identified as a result of binding of the labelled lectin and included p30 and p32. The failure to identify those components as glycoproteins previously, using the galactose oxidase labelling method, suggests that they contain carbohydrate chains of the B type, i.e. those which have mannose and glucosamine residues only (Shiraishi et al. 1977). The two remaining major polypeptides p64 and p23 were not found to be glycosylated, by either method.

Non-ionic detergents and bile salts have been used extensively to dissociate viral and other membranes into soluble complexes that retain biological activity. Simons et al. 1973, disrupted the envelope of Semliki Forest virus by treatment with Triton X-100 into soluble protein and lipid complexes, which were then separated by density gradient centrifugation in the presence of the detergent. Hayman et al. (1973) successfully isolated the glycoproteins of influenza and mouse mammary tumour viruses by affinity chromatography using columns of immobilized phytohaemagglutinin equilibrated with buffer containing sodium deoxycholate. Of considerable advantage in both studies was the retention of the biological activities of the membrane glycoproteins after virus disruption. In the studies reported here,
purified HBsAg was disrupted with 2% Triton X-100 in the presence of salt to give a product with an estimated sedimentation coefficient of 3.9S (Fig. 1). Disrupted HBsAg was then fractionated by passage through a column of immobilized concanavalin A in the presence of Triton X-100 (Fig. 4). The first fraction, which did not bind to the lectin, contained exclusively the polypeptide p64. The co-migration by SDS-PAGE of p64 with serum albumin run on a parallel gel prompted further studies as to the exact nature of this component. It was found that p64 reacted with an antiserum to human serum albumin (Table 1) and produced a similar peptide map to that of purified chimpanzee albumin (Fig. 6). These findings clearly demonstrated that serum albumin was present in purified preparations of HBsAg small particles. The question was considered as to whether this was a residual contaminant or a constituent of the particle itself. The latter interpretation appeared the more likely for the following reasons: firstly, it was calculated that the dilutions of the original serum at each stage of purification would result in a final concentration of albumin about 100 times lower than the concentration of p64 actually found. Secondly, affinity chromatography of purified HBsAg on a column of Blue Sepharose CL-6B, an effective method of removing albumin from serum (Travis & Pannell, 1973), resulted in the binding of the antigen to the column in conditions identical to those required for the binding of albumin (Fig. 7). Finally, purified HBsAg was clumped by anti-albumin antibodies as demonstrated by electron microscopy (data not shown). These findings are indicative of a close association between serum albumin and HBsAg 20 to 25 nm particles. It is noteworthy in this context that the appearance of auto-antibodies to albumin has been described as a feature of hepatitis B infection (Lenkei et al. 1977); in addition it has been suggested that HBsAg from persistently infected individuals may contain receptors for polymerized albumin. (A. R. Neurath, personal communication.) It is conceivable that the close association of albumin with HBsAg determinants may stimulate an auto-immune response to this host protein during hepatitis B virus infection.

The second fraction obtained from the concanavalin A-Sepharose column by elution with α-MM contained p28 and p23 as major components. The finding that p23 does not bind concanavalin A when separated in an SDS-polyacrylamide gel, suggests that the polypeptide may be joined to p28 by a protein–protein linkage unaffected by detergent treatment. The reactivity of this material with antibodies to surface antigen, in the absence of any significant reactions with antibodies to the normal serum constituents, indicates that HBsAg reactivity resides with this subunit preparation. Studies are currently in progress both to examine the immunogenicity of this material and to compare its efficiency with that of intact 20 to 45 nm particles for the protection of susceptible chimpanzees from hepatitis B infection. The technique of Triton X-100 solubilization followed by affinity chromatography also allows for the preparation of milligram quantities of serologically reactive material which has not been possible using preparative SDS-PAGE methods.

These studies provide a basis for an alternative method of preparing a subunit vaccine for the immunoprophylaxis of hepatitis B and demonstrate that purified HBsAg 20 to 25 nm particles contain significant quantities of serum albumin. The removal of this and any other host-coded components may be desirable before the introduction of a vaccine prepared from the serum or plasma of individuals persistently infected with hepatitis B virus.

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