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The structure of HBsAg particles is not modified upon their adsorption on aluminium hydroxide gel☆

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ABSTRACT

Current Hepatitis B vaccines are based on recombinant Hepatitis B surface antigen (HBsAg) virus-like particles adsorbed on aluminium (Al) gel. These particles exhibit a lipoprotein-like structure with about 70 protein S molecules in association with various types of lipids. To determine whether the adsorption on Al gel affects HBsAg structure, we investigated the effect of adsorption and mild desorption processes on the protein and lipid parts of the particles, using various techniques. Electron microscopy showed that the size and morphology of native and desorbed HBsAg particles were comparable. Moreover, infrared and Raman spectroscopy revealed that the secondary structure of the S proteins was not affected by the adsorption/desorption process. Affinity measurements with Surface Plasmon Resonance showed no difference between native and desorbed HBsAg for HBsAg-specific RF-1 monoclonal antibody. Steady-state and time-resolved fluorescence data of the intrinsic fluorescence of the S proteins further indicated that the adsorption/desorption of HBsAg particles on Al gel did not modify the environment of the most emitting Trp residues, confirming that the conformation of the S proteins remains intact. Moreover, using environment-sensitive 3-hydroxyflavone probes, no significant changes of the lipid core and lipid membrane surface of the HBsAg particles were observed during the adsorption/desorption process. Finally, the ratio between lipids and proteins in the particles was found to be similar before and after the adsorption/desorption process. Taken together, our data show that adsorption on Al gel does not affect the structure of the HBsAg particles.

1. Introduction

Hepatitis B virus (HBV) infection is one of the most common human diseases. Each year, over one million people die from HBV-related chronic liver diseases [1,2], explaining the importance of Hepatitis B vaccination. Currently, Hepatitis B vaccines are based on recombinant HBsAg particles produced in yeast cells [3,4] or mammalian cells [5–7] and adsorbed on aluminium (Al) gel. HBsAg particles are 22-nm spherical particles composed of host cell-derived lipids and up to three virus-encoded glycoproteins, designated as large, medium and small (L, M, S). Compared to mammalian-derived HBsAg particles, yeast-derived particles, like the *Hansenula polymorpha*-derived HBsAg particles used in this study, contain only unglycosylated S proteins. This protein is highly hydrophobic [8] and in tight association with lipids in the HBsAg particles [6,7]. The lipid part of the HBsAg particles is mainly composed of phospholipids [4,9,10]. Lipids have been shown to be involved in the antigenic properties of HBsAg particles [11], stabilizing their structure and the protein conformation [4]. Several studies characterized the lipid/protein organization of the HBsAg particles [4,6,8,9,12–14] and we recently proposed a model for the HBsAg structure [15]. The particle is thought to exhibit a lipoprotein-like structure with an ordered and rather rigid lipid interface and a more hydrophobic and fluid inner core. About 70 proteins are included in each particle with a part protruding out [9,16] and another deeply inserted in the lipid core [15].

Al hydroxide or phosphate are the most common adjuvants for human and veterinary vaccines [17], used to potentiate immune responses, enhancing antigen uptake [18] and stimulating

Abbreviations: Al, aluminium; CD, circular dichroism; EM, electron microscopy; HBsAg, Hepatitis B surface antigen; HBV, Hepatitis B virus; IR, infrared; PLGA, poly (D,L)-lactide-co-glycolide acide; RAMFc, rabbit anti-mouse IgG Fcγantibody; SPR, Surface Plasmon Resonance.

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immune-competent cells [19,20]. Despite this widespread use, their properties and interactions with antigens have been poorly investigated. In vaccines against HBV, the adsorption of the HBsAg particles on the Al gel results from the binding of the phosphate groups of the phospholipids from HBsAg surface with the hydroxyl groups of the Al gel [21] through a ligand-exchange mechanism. When the vaccine is administered upon intramuscular or subcutaneous injection, a part of the antigens adsorbed to Al gel is expected to be eluted when it comes in contact with interstitial fluid [22,23].

The induction of a strong immune response is dependent on the integrity of the antigens. Since epitopes are conformational in nature, the structure of protein antigens on the surface of Al gel needs to be better characterized [24]. Recently, antigens after Al adsorption have been characterized by monitoring their conformation on Al hydroxide [25–27] or after desorption from the adjuvant surface [28]. Since antigens are often strongly bound to the adjuvant, drastic methods of elution using surfactants [29] or extreme pH changes [30] have been used but were found to lead to alteration of the desorbed antigens. Only recently, a soft method of desorption using competing phosphate anions has been developed [31].

For HBsAg particles, an alteration of their structure after adsorption on Al gel was described [27], but the adsorbed particles were only observed by immunoelectron microscopy after denaturing size exclusion chromatography and sodium dodecyl sulphate-polyacrylamide gel electrophoresis in reducing conditions.

In this study, we compared with various complementary techniques, the structure of native and adsorbed HBsAg particles with that of HBsAg particles desorbed with a soft method in non-denaturing conditions. Altogether, our results show that the structure of HBsAg particles is not modified by adsorption/desorption process.

2. Materials and methods

2.1. Materials

HBsAg virus-like particles were produced by Sanofi Pasteur in the recombinant yeast *Hansenula polymorpha* and obtained in a highly purified form after successive steps of fermentation, extraction and purification. Aluminium gel (Alhydrogel, Superfos) was purchased from Brenntag Biosector. RF-1 mAb was purchased from the NIBSC (National Institute for Biological Standards and Control). FN4 and PPZ8 dyes were synthesized as described elsewhere [32–34].

2.2. Adsorption/desorption process

The protocol of adsorption/desorption of HBsAg particles was derived from that of Egan et al. [31]. At room temperature, Al gel (6 mg Al/mL) was mixed with HBsAg particles (324 μg/mL) in phosphate buffer (30 mM) at pH 7.4. The sample was mixed gently by end-over-end rotation for 30 min to obtain complete adsorption. The sample was then centrifuged at 1000 rpm during 5 min to pellet the adsorbed material. In order to softly desorb the particles from the gel, 400 mM of phosphate buffer was added to the pellet. The sample was mixed during 1 h, and centrifuged at 3000 rpm during 5 min. HBsAg particles in the supernatant are referred to desorbed particles. The percent of HBsAg particles released after the desorption process was found to be about 10 ± 2%, in excellent agreement with the original paper on this method [31].

2.3. Electron microscopy (EM)

For negative staining, HBsAg solutions (native and Al-desorbed) were diluted in Tris 10 mM, NaCl 150 mM buffer, pH 7.4 to a concentration of 20 μg/mL, deposited on a 400 mesh full carbon-coated glow-discharged grid and stained with 2% uranyl acetate [16]. EM measurements were performed with a JEOL JEM 2100F transmission electron microscope, as previously described [16].

2.4. Infrared spectroscopy (IR)

HBsAg samples (native and Al-desorbed) were concentrated using capped centrifuge device (Amicon, Millipore) with a cut-off of 5 kDa up to 1.8 mg/mL in protein.

Fourier transform infrared (FTIR)-transmission spectra were recorded with a Vector 70 spectrophotometer (Bruker, Germany) equipped with a highly sensitive photovoltaic MCT detector cooled with liquid nitrogen. The spectrophotometer was continuously purged with dry N_2 to remove water vapour. In addition, an automatic water vapour correction was performed using the Opus software 5.5 version (Bruker, Germany). The Aqua Spec accessory (Bruker, Germany) consisting in a transmission cell with a path length of 6.8 μm between CaF_2 windows, has been used. Spectra were obtained from the collection of 120 scans per sample at a resolution of 4 cm^{-1} and were corrected from buffer. Protein secondary structures were calculated using the Confochek system (Brucker). Second-derivative spectra were obtained using the Opus software 2.2 version with a nine-point Savitsky-Golay derivative function.

2.5. Circular Dichroism (CD)

Far-UV CD spectra of native, adsorbed and desorbed HBsAg particles were recorded on a Jasco-810 spectropolarimeter in a 0.1 cm path-length circular cell (Hellma). To avoid sedimentation, the 0.2 mg/mL protein-containing samples were rotated at 52 rpm. Spectra were recorded at room temperature by averaging 3 scans from 280 to 180 nm (1 nm bandwidth) in 0.5 nm steps at a rate of 50 nm/min, and 1 s response. Blank spectra of either aqueous solutions or Al gel were used to correct the observed spectra. Data were analysed and smoothed by the means-movement method using the Jasco Spectra Analysis software. Spectra from 197 to 260 nm were subjected to secondary structure analysis using the Jasco CD Multivariate SSE software.

2.6. Surface Plasmon Resonance (SPR)

Antigen-antibody interactions were investigated with a temperature-controlled BIAcore 3000 instrument (Upsala, Sweden). The running buffer contained 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.005% (w/v) polysorbate 20. The flow rate was maintained at 70 μL/min, except during acid regeneration where the flow rate was 30 μL/min. Affinity-purified polyclonal rabbit anti-mouse IgG Fcγ antibody (RAMFc) was chemically immobilized on the CM5 sensor chip surface using an amine coupling kit (GE). The HBsAg-specific mAb RF-1 [35] was captured by RAMFc (80RU), and the affinity of HBsAg particles towards RF-1 mAb was determined by injecting different concentrations of HBsAg particles onto the sensor chip. The kinetic parameters and the equilibrium dissociation constant were determined using the BiaEval software.

2.7. Fluorescence spectroscopy

All experiments were performed in 8 mM phosphate buffer saline (PBS), NaCl 150 mM, pH 7.4, at 20 °C. Concentration of proteins for HBsAg particles was 0.4 μM, except for quantum yield measurements, where it was 2 μM. As the gel induces strong light scattering, time-resolved fluorescence decay, quantum yield, KI quenching and steady-state anisotropy could not be measured for the adsorbed particles. Characterization of the lipid and S proteins organization was performed, as previously