COMUNICACIÓN CORTA / SHORT COMMUNICATION

Characterization nanoparticles-based vaccines and vaccine candidates: a Transmission Electron Microscopy study

Caracterización por Microscopía Electrónica de Transmisión de vacunas y candidatos vacunales basados en nanopartículas

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ABSTRACT

Transmission Electron Microscopy (TEM) is a valuable tool for the biotech industry. This paper summarizes some of the contributions of MET in the characterization of the recombinant antigens are part of vaccines or vaccine candidates obtained in the CIGB. It mentions the use of complementary techniques MET (Negative staining, and immunoelectron) that enhance visualization and ultrastructural characterization of the recombinant proteins obtained by Genetic Engineering.

Keywords: TEM, HBV, NASVAC, Dengue, negative staining, nanoparticles.

RESUMEN

La Microscopía Electrónica de Transmisión (MET) constituye una herramienta valiosa para la industria Biotecnológica. Este trabajo resume algunos de los aportes de la MET en la caracterización de los antígenos recombinantes que forman parte de vacunas o candidatos vacunales obtenidos en el CIGB. Se hace mención al uso de técnicas complementarias a la MET (Tinción Negativa, y la Inmunomicroscopía) que potencian la visualización y caracterización a nivel ultraestructural de las proteínas recombinantes obtenidas por Ingeniería Genética.

Palabras clave: MET, VHB, NASVAC, Dengue IME, Tinción Negativa, Nanopartículas.

Introduction

The necessity to develop effective drugs against infectious diseases has made electron microscopy a very important tool in the field of Biotechnology. Specifically, the Transmission Electron Microscopy (TEM) contributes to the development of new methodologies in the diagnostic of viral diseases and add new elements to study the mechanisms involved in the pathogenesis of the diseases [1]. The combination of TEM and Immunoelectron Microscopy (IEM) has also made the visualization and characterization, at ultrastructural level, of recombinant proteins obtained by genetic engineering possible.

TEM is based on the incidence of a beam of electrons, produced by a cathodic filament, through a sample, and the electronic scattering caused by the atoms composing the structure of the sample [2]. TEM has a resolution in the order of the nanometers, higher than the resolution of the optic microscopy.

TEM has extensively been used in the research and characterization of biotechnological products for human and plant health, based on DNA recombinant techniques, at the Center for Genetic Engineer and Biotechnology (CIGB) in Cuba. For example, using this method, different systems for the expression of recombinant proteins and diverse methods for the detection of viruses and recombinant proteins in bacteria, yeast and plants have been analyzed.

In this work, we highlight the more important contributions of TEM in the characterization of the nanoparticles conforming the vaccines and vaccine candidates for human and animal uses.

Methods

Negative staining Samples were analyzed using the method. Briefly, the samples were centrifuged 20 minutes, contrasted with uranyl acetate and visualized with a Transmission Electron Microscope Jeol JEM 2000 Ex.

Immunoelectron Microscopy: The samples were incubated with either CBSS-HepB.1 mAb in phosphate buffer, for 45 min at RT. The samples were rinsed three times for 30 min at RT with 0.1% bovine serum albumin in phosphate-buffered saline pH 7.3 (BSAPBS),and incubated for 1 h at RT with gold-labeled (15nm) anti-mouse IgG (Amersham, UK) diluted 1:100 in BSA-PBS. As control the primary antibody was substituted by normal mouse serum.

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Development

The distribution, the morphology and the particle size are important parameters in evaluating the pharmaceutical composition based on virus-liked particles (VLP). Nowadays, TEM is still one of the most important analytical tools for the ultrastructural analysis of antigens and proteins (in vaccines and vaccine candidates) obtained by recombinant technology. Different methods have been combined with TEM for the identification and direct classification of viral particles and the detection of strange agents and other contaminants in the samples. Nevertheless, the negative staining method has been preferred because of its simplicity and fidelity [3]. IEM is one of the most important techniques in the detection and localization of proteins in cells and tissues. This method is based on the detection of the antigen-antibody interaction using a secondary antibody labelled with colloidal gold particles [4]. The sensitivity of TEM highly increases when this method is combined with the IEM.

At CIGB, one of the more important applications of TEM is the characterization of nanoparticles conforming vaccines and vaccine candidates. These nanoparticles can be formed by proteins, protein/lipid or protein/DNA complexes, and they have the property of mimicking supramolecular structures of viruses. They can also induce an antiviral immune response, in terms of neutralizing antibodies and/or a cell-mediated immunity (CMI) [5]. One of the main characteristics of nanoparticlesis is their high degree of particulation and/or aggregation caused by covalent and non-covalent molecular interactions. This characteristic allows them to be used as vaccines. [6] TEM allows the evaluation of the stability, the homogeneity, and the consistency of the particles in the formulation of the production processes of the Hepatitis B and cattle tick vaccines. Moreover, particles composing the vaccine candidates being developed in our institution are also characterized. The Cuban vaccines against Hepatitis B (Heberbiovac®) and the cattle tick (Gavac®) are distinctive products of CIGB in the biomedical and agricultural departments.

Hepatitis B is a deadly disease caused by the hepatitis B virus. It is a very important global health problem, causing around 600 000 deaths per year [7] therefore, the development of effective vaccines against the virus is mandatory. Since 1992, the CIGB produces a recombinant vaccine against the Hepatitis B virus. During these years, more than 100 million of doses of this prophylactic vaccine have been distributed in 35 countries. AgsHB is the active pharmaceutical ingredient (API) of the cuban Hepatitis B vaccine, Heberbiovac®, which is produced in the yeast *Pichia pastoris*.

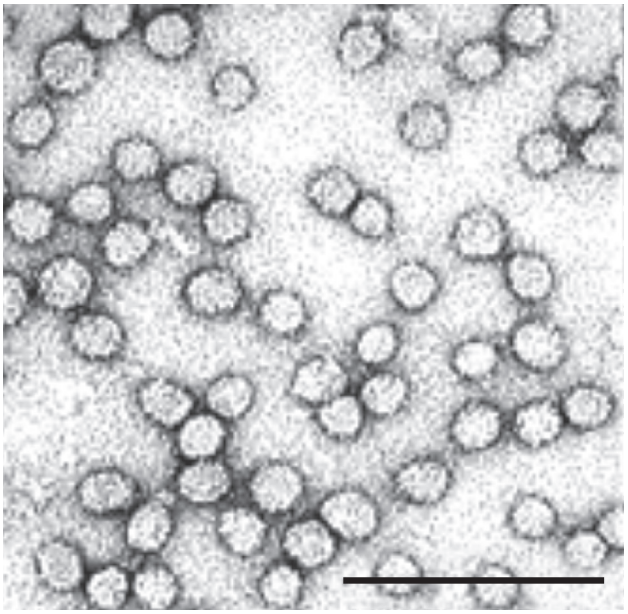


Fig. 1. Vaccine Heberbiovac. Presence of recombinant nanoparticles is observed, approximately 20nm in diameter. Bar = 100nm.

On the other hand, *Boophilus* ticks are the most harmful ectoparasites affecting cattle in Latin America, Australia and some regions from Africa. They cause stress and weakness of the affected animals. From ticks per animal, the damage has economic effects: decrease of the animal weight and milk production, negative effects on the fertility and increase the incidence of other diseases. For this reason, the development of effective vaccines against this disease is crucial [8]. The protein Bm86 is the API of the Cuban vaccine against the cattle ticks (Gavac®, CIGB, Cuba) produced in the yeast *Pichia pastoris*.

The importance of TEM as quality specification criteria for the release of the Gavac and AgsHB batches during their production processes has been demonstrated. The effectiveness of both vaccines has been related to their high degree of particulation. The experimental results suggest that this characteristic increases their immunogenicitiy [9,10]. Using TEM and negative staining, the particulated properties of AgsHB and Bm86 were corroborated (Fig. 1 and 2). These nanoparticles were also analyzed using IEM with monoclonal antibodies highly specific against the APIs (Fig. 3).

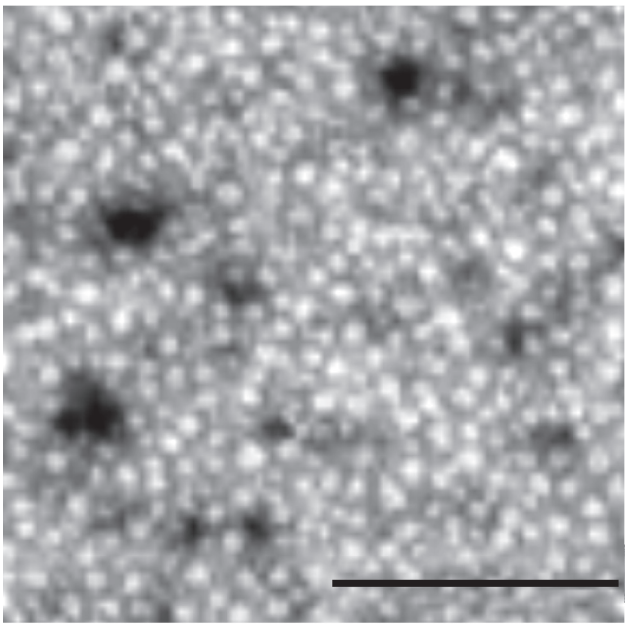


Fig. 2. Vaccine Gavac. Presence of recombinant nanoparticles is observed, approximately 20nm in diameter. Bar = 200nm.

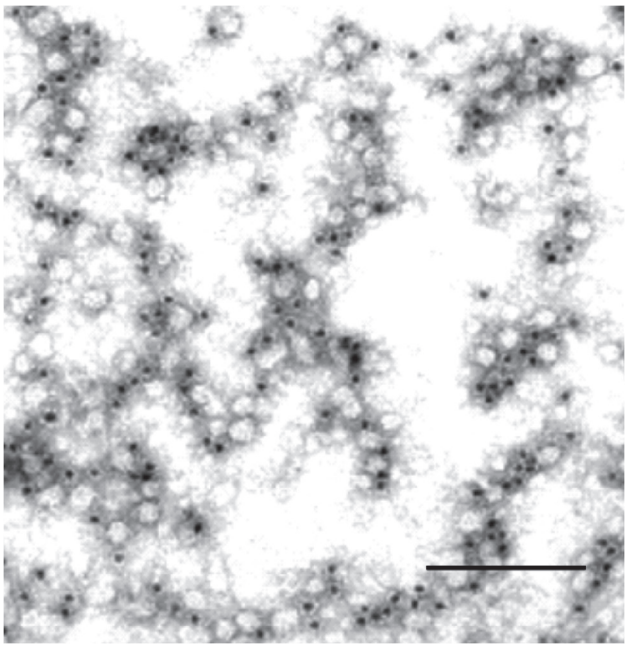


Fig. 3. Vaccine Heberbiovac (Immune Electron Microscopy). Bar =200nm.

Currently, in our institution there are research projects investigating the development of new vaccines against infectious diseases like Hepatitis B and dengue. The new vaccine candidate against hepatitis B (still in developmental process) is focused on a therapeutic use unlike the previous Heberbiovac that has a prophylactic use. The name of this new future product is NASVAC and it is based on the combination of the surface antigen (HBsAg) and the nucleocapside of the hepatitis B virus (HBcAg). Both antigens have sizes similar to the virus: 22-26 nm. This is a key element for their interaction with the immune system and the development of an effective antiviral response.

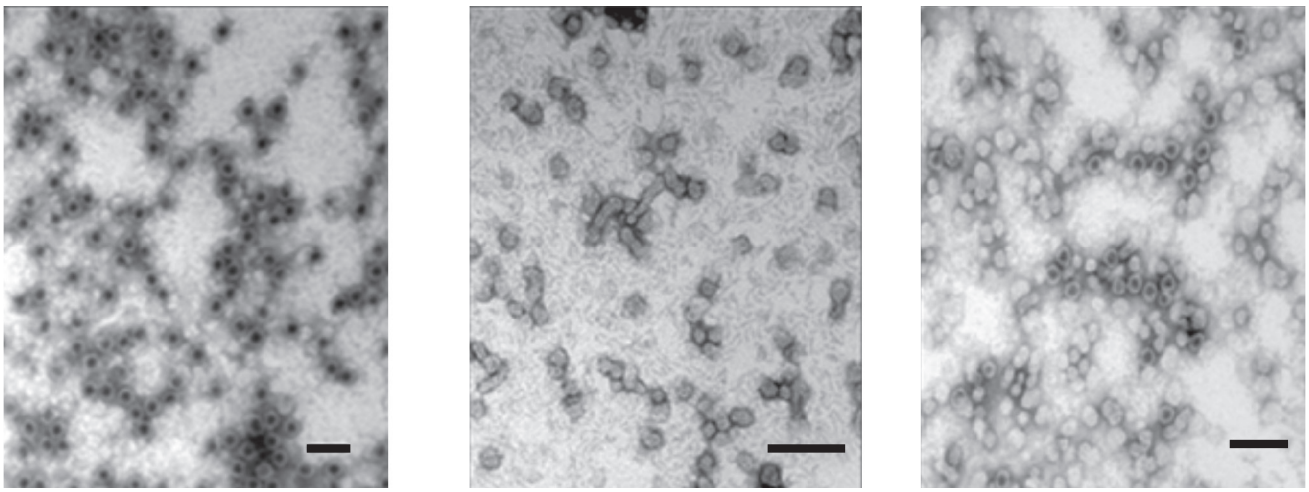
Therefore, it is important to obtain, after the production processes, homogeneous particles for each antigen. TEM directly enables the identification and characterization of sizes and the morphology of these particles.

Previous studies suggested that some features such as high degree of particulation and aggregation could increase the immunogenicity of the vaccines. Aguilar et. al (2004) also demonstrated that the adhesiveness of the recombinant antigens HBsAg and HBcAg, favor the formation of particulate aggregates in a range between 22-200 nm. Because of this characteristic, the AgcHB provokes a stronger lymphocytes activation. TEM analysis confirms the presence of these particles in different formulations of the vaccine candidate Nasvac (Fig. 4).

Dengue is the most important viral disease transmitted by arthropods affecting the world population. Every year 50-100 millions of new cases of this disease are reported and 500 000 of them, correspond to the hemorrhagic denguefever, which is the most severe manifestation of the disease. This disease is produced by the dengue virus (DV), which is a viral complex composed of 4 serotypes. Unfortunately, there is not an effective vaccine against dengue. In Cuba, there is a research program for obtaining a subunit vaccine against the four serotypes of the virus. Using TEM it was possible to characterize one of the recombinant proteins constituting the base of the vaccine candidate. This protein, named DIIIC-2, is composed by the domain III of the viral envelope protein and the capsid protein of the dengue-2 virus. These viral regions are antigenic fragments able to induce humoral and cellular immune responses against the virus.

TEM with negative staining demonstrated the presence of particles after the incubation of the protein DIIIC-2 with oligonucleotides of unknown DNA sequence. Valdes et. al. (2009), Gil et. al. (2009) and Marcos et. al. (2013) also demonstrated that after the immunization with these aggregated structures, an immune cellular response and neutralizing antibodies were induced. This response had a protective capacity in mice. It was also demonstrated that the formulation containing the non-aggregated protein did not induce a functional immune response (Fig. 5).

Plants have also been important biotechnology models for the production of recombinant proteins. TEM analysis revealed the presence of spherical viral particles of 27 nm similar to the Hepatitis A virus (HAV) in purified samples from transgenic



A B C

Fig. 4. Presence of recombinant nanoparticles of AgcHB (a), AgsHB (b) and the combined formulation of the Vaccine Candidate NASVAC (c). Bar=100nm.

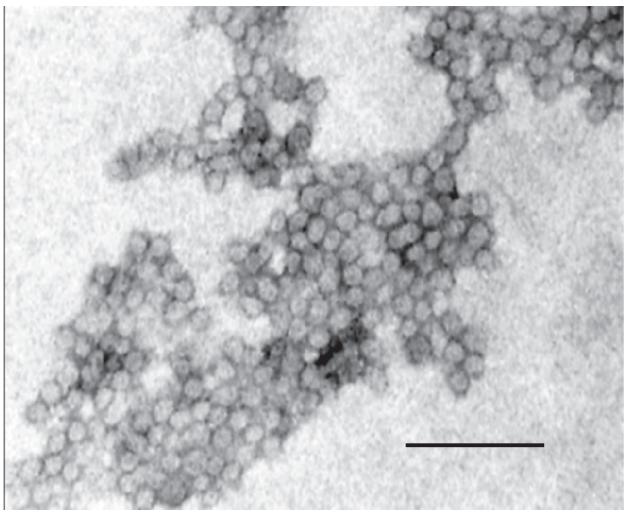


Fig. 5. Vaccine Candidate against Dengue virus. Presence of recombinant nanoparticles is observed, approximately 20nm in diameter. Bar =100nm.

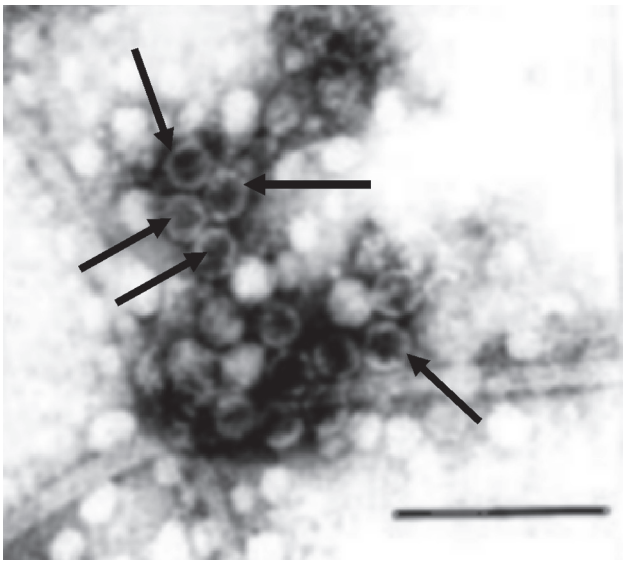


Fig. 6. Vaccine Candidate against VHA virus. The presence of recombinant nanoparticles is observed, approximately 27nm in diameter. Bar = 200nm

tobacco plant expressing HAV open reading frame (11). It was the firstreport of the production of this antigen in plant being the formation of these particles required to obtain a stronger immune response (Fig. 6).

Conclusions

TEM constitutes a very powerful biotechnological tool for the study of the recombinant nanoparticles. Specifically, we demonstrate here how useful is this method for detecting aggregation states of the nanoparticles conforming the vaccines and vaccine candidates produced and under developed at CIGB. Our group has obtained experiences very useful for the future.

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