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Evaluation of uptake and generation of immune response by murine dendritic cells pulsed with hepatitis B surface antigen-loaded elastic liposomes

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Abstract

Hepatitis B surface antigen (HBsAg)-loaded elastic liposomes were studied for qualitative and quantitative uptake *in vitro* by murine dendritic cells (DCs) generated from bone marrow mononuclear cells. Internalization of the vesicles by the DCs was documented using fluorescence microscopy. Kinetics of uptake of antigen-loaded elastic vesicles by the DCs studied through flow cytometry showed a peak uptake at 6 h. The ability of the antigen pulsed DCs to stimulate autologous peripheral blood lymphocytes was demonstrated by BrdU assay. Further evaluation by multiplex cytometric bead array analysis demonstrated a predominantly TH1 type of immune response. Our results suggest that HBsAg-loaded elastic vesicles as antigen delivery module and DCs as antigen presenting cells are able to generate a protective immune response. The property of elastic liposomes to traverse and target the immunological milieu of the skin makes it an attractive vehicle for development of a transcutaneous vaccine against hepatitis B virus.

Keywords: Elastic vesicles; Hepatitis B vaccine; Dendritic cells; Transcutaneous immunization

1. Introduction

Hepatitis B is a small DNA virus that belongs to a group of hepatotropic DNA viruses. Hepatitis B virus (HBV) infection is a global public health problem, with approximately 400 million people being chronically infected. Each year it causes more than 500,000 deaths worldwide. Infection with the virus leads to a wide spectrum of clinical presentations ranging from an asymptomatic carrier state to self-limited acute or fulminant hepatitis to chronic hepatitis leading to cirrhosis and hepato-cellular carcinoma [1]. Hepatitis B vaccination is the most effective measure to prevent HBV infection. Universal hepatitis B immunization has also been shown to be highly cost-effective in low-income countries with intermediate endemicity [2]. The available vaccines produce good and sustained immune response however the effectiveness falls with age and they are less effective in special groups like patients with chronic renal failure, liver transplant recipients and HIV-affected individuals [3].

Dendritic cells (DC) are specialized antigen presenting cells (APCs), which have attracted much attention by their ability to initiate a robust T-cell-dependent immune response. DCs are very efficient in capturing, processing and presentation of antigens with stimulation of naïve T lymphocytes and are even capable of priming naïve $CD8^+$ T lymphocytes [4]. DCs are leukocytes distributed throughout lymphoid and non-lymphoid tissues, in peripheral blood and afferent lymph vessels and are localized at strategic places in the body at sites used by pathogens to enter the organism [5–7]. There are now reproducible ways to cultivate DC *in vitro* from peripheral

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blood mononuclear cells or bone marrow derived stem cells. DCs, after activation, express high levels of several molecules on the cell surface such as MHC classes I and II, accessory molecules CD40, CD80, CD86 and early activation markers such as CD83. The ability of DCs to invoke a primary response is due to expression of cell surface markers along with co-stimulatory signals, which are essential for T-cell activation.

Elastic liposomes, the ultradeformable carriers for potential transcutaneous application, contain a mixture of lipids and biocompatible membrane softeners. The optimal mixture leads to flexibility of the elastic liposomes membranes and to the possibility of penetration through channels of the skin, which are opened by the carriers [8–10]. It has also been shown to be useful for non-invasive vaccination and would be ideal for large immunization program [11].

A transdermal delivery system offers an interesting module to approach DC subsets in order to enhance immunity against viral infection, cancer or pathogens. The immune system of the skin harbors two very potent antigen presenting DC subsets, which induce primary antigen-specific T-cell immune responses [5]. Transcutaneous immunization (TCI) is a novel needle free immunization method developed for delivery of vaccines. It involves the application of antigen and adjuvant onto the skin to induce antigen-specific immune responses. In the past, the skin was seen as a barrier through which vaccines could not be delivered. Nowadays the skin, an immunocompetent organ, has been proven to be suitable for vaccine delivery [12,13].

Previously we have reported elastic liposomes bearing HBsAg provides higher entrapment efficiency, enhanced penetration and effective immuno-adjuvant property as compared to conventional liposomes, topically applied plain HBsAg solution and physical mixture of HBsAg and elastic liposomes justifying its potential for improved vaccine delivery. We also compared the formulations in terms of antibody titer (humoral response) with intramuscularly administered alum adsorbed HBsAg solution and observed comparable systemic IgG and secretory IgA responses [14]. In the present study, DCs generated from mouse bone marrow cells were studied for their uptake of elastic liposomes by flow cytometry and spectral bio-imaging. Additionally the immune responses elicited *in vitro* by these pulsed DCs were studied by BrdU proliferation assay and the TH1/TH2 response was studied using multiplex cytometric bead array analysis.

2. Materials and methods

2.1. Materials

The hepatitis B surface antigen (HBsAg; source-genetically modified yeast cells) was obtained as gift sample from the National Institute of Immunology, New Delhi, India; Albumin bovine (fraction V), Fluorescein isothiocyanate (FITC), rhodamine, Soya phosphatidyl choline (SPC) were purchased from Sigma, USA. RPMI-1640, Alamar blue, foetal calf serum and sodium azide were procured from Hyclone, USA. Annexin-V-Fluos staining kit and BrdU were obtained from Roche Applied Sciences, Germany. FACS buffer, Granulocyte macrophage colony stimulating factor (GM-CSF) and IL-4 were purchased from BD Biosciences, USA.

2.2. Preparation of vesicular system

Elastic liposomes were prepared by method as described previously [14]. In brief, ethanolic solution of Soya Phosphatidyl Choline was mixed with Span 80 (86:14%, w/w) in phosphate buffer (pH 6.5) containing HBsAg solution (10 μg/ml). Similarly FITC-BSA and rhodamine B-BSA-loaded elastic liposomes were prepared for Spectral bio-imaging and FACS, respectively. The obtained suspension was extruded through a sandwich of 0.45, 0.22 μm polycarbonate filters (Millipore, USA).

2.3. Isolation and culture of dendritic cells

DCs were generated from murine bone marrow cells as reported elsewhere [15–17]. DCs were generated from murine bone marrow cells by washing out the bone marrow two to three times and cells were plated in six-well tissue culture plates (BD Discovery Labware, USA) at 2×10^6 cells per well in 10 FCS RPMI-1640. After incubation for 1 h at 37 °C/5% CO_2, nonadherent cells were removed by gentle washing and adherent cells were cultured with RPMI-10 FCS enriched with 500 U/ml GM-CSF and 8 ng/ml IL-4 to generate DCs. On days 3 and 6 culture media was replaced.

2.4. Flow cytometric analysis for evaluation of kinetics of uptake

On day seven of culture, 100 μl of elastic liposomes containing HBsAg (rhodamine labeled) were added to wells. Kinetics of uptake of the system by the cells was studied at 0, 1, 3, 6, 12 and 24 h time intervals. At the end of each interval the cells were harvested, excess of formulations was removed by washing with ice-cold PBS containing 0.01% sodium azide and 5% foetal calf serum, and resuspended in FACS buffer. Phagocytosis was measured by flow cytometer (BD FACSCalibur, USA) equipped with an argon ion laser at an excitation wavelength of 488 nm. For each sample, 10,000 events were collected. Cell-associated rhodamine was measured by Cell Quest Software (BD-IS, USA) and in some cases the cells were double labeled with FITC conjugated antibody (anti-CD11c mAb) to confirm the gating for DC. Mock treated DC (DCs pulsed with elastic liposomes not loaded with antigen) was taken as control [18].