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Immunization with functionalized carbon nanotubes enhances the antibody response against mode antigen ovalbumin

Xinfeng Zhu, Jiadi Sun, Yinzhi Zhang, Xiulan Sun*

State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Synergetic Innovation Center of Food Safety and Nutrition, Jiangnan University, Wuxi, Jiangsu 214122, China

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ABSTRACT

Carbon nanotubes (CNTs) have attracted considerable attention because of their potential application as a new nonvehicle. We have covalently conjugated the mode antigen ovalbumin (OVA) to functionalized multi-walled carbon nanotubes. Herein, we explored the underlying theoretical mechanisms of CNTs' immunological adjuvant characterization. In vitro, the efficiency of cellular uptake of MWCNT-OVA into DC2.4 cells was improved over that of pure-antigen OVA. The costimulators (CD40/86), the major histocompatibility complex MHCII molecules, and the CD11c molecules were found to be upregulated. Further in vivo experiments established that the MWCNT-OVA group enhanced the IL-1β, TNF-α, and IL-6 cytokine secretion, suggesting that MWCNT reinforced the immune response using different cytokine pathways. Anti-OVA antibodies after inoculation of MWCNT-OVA into mice were measured. The medium dose of MWCNTs conjugated with OVA induced the highest level of OVA-specific antibodies at day 82 and have a synergistic effect with the commercial Freund's adjuvant. MWCNTs-KLH-MC-LR also induced higher levels of MC-LR-specific antibody than did KLH-MC-LR. MWCNTs also could activate the complement system which is closely related with humoral immunity. These results suggested that MWCNTs enhance the immune response and show excellent inherent characteristics to be applied as an adjuvant.

1. Introduction

Carbon nanotubes (CNTs) [1] are rolled hexagonal carbon networks capped by half fullerene molecules. Single-walled (SWCNTs), double-walled (DWCNTs), and multi-walled (MWCNTs) are three common carbon nanotube configurations. Due to their unique physical and chemical properties, carbon nanotubes are attracting increasing attention in the biological field as new vectors for the delivery of biomolecules such as peptides, proteins, and nucleic acid [2–6]. CNTs' role in modulating immunological functions is one area of particular interest. CNTs have a relatively large surface area for chemical modification and can carry a large amount of antigen. The adjuvant effect of CNTs on immune responses has been shown to increase when CNTs' size is decreased and surface area is increased. Conjugation of antigenic epitopes to nanoscale and submicroscale particulate scaffolds has shown that the particles improve immune responses [7]. Immunogens, such as tumor lysate protein [8] and viral peptides [9], conjugated to CNTs can cause strong antibody responses in mice and no detectable cross reactivity to the CNTs was found.

Peptide presentation by antigen presenting cells (APCs) is generally necessary for the generation of an immune response. Functionalized carbon nanotubes have been shown to cross cell membranes and to accumulate in the cytoplasm without being toxic to the cell [12]. CNT can be up taken by a wide variety of cell types and through several mechanisms, which appear to be particularly suited to deliver antigenic epitopes to APCs. Antigen delivery through nanoparticles also changes cellular trafficking and can acts as an intracellular depot of antigen, both of which activities enhance the immune response to the delivered antigen [13].

Nanoparticles alone can induce antibody production and cytokine secretion [14–16]. APCs such as macrophages and dendritic cells, phagocytose external materials and promote lymphocytes and other immune cells by releasing cytokines, initiating an adaptive immune response [2,17]. Hence, it is essential to further explore nanoparticles' application to adjuvant activity in order to understand the effects of nanoparticles on cytokine release [18,19].

The humoral immunity is closely related with the complement system. The complement system consists of more than 30 species of protein which are widely found in serum, tissue fluid, and cell surface, and which participate in the innate immune system and

* Corresponding author.
E-mail addresses: zxfyiliya@163.com (X. Zhu), sxlzzz@jiangnan.edu.cn (X. Sun).

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adaptive immunity. The complement system could be able to identify, opsonize, and remove or kill foreign materials such as invading microorganisms and altered host cells. CNTs and functionalized CNTs can impact the immune system by activating the complement system [10,11].

However, there are few systematic explorations of CNTs' effect on activation of specific immunity and the underlying theoretical mechanisms. In this paper, we focus on the immune response induced by MWCNTs. In vitro, we employed DC2.4, which are the most effective antigen presenting cells with which to study antigen uptake and antigen-presenting ability in cell-based experiments. In vivo, the levels of anti-OVA antibody production in mice were compared after immunization with MWCNT-ova and with MWCNTs alone. We asked whether MWCNT could act as complement activators. We found that antibody production is significantly increased in mice after immunization with MWCNT – OVA. To investigate the theoretical view of the NP-based adjuvant, we tried to build a bridge between the in vitro and in vivo performance. In addition, the antibody response induced by MWCNT co-administered with the conventional adjuvant Freund's adjuvant was studied. In addition, the synthesis of a microcystin-LR-KLH- MWCNT was also made to verify the increased antibody response induced by MWCNTs for small molecule antigens.

2. Materials and methods

2.1. Materials and reagents

Dulbecco's modified Eagle's medium (DMEM), RPMI-1640, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from GIBCO. HRP-conjugated anti-mouse IgG were obtained from Jackson. Fluorescence-labeled anti-mouse anti-I-A[b] MHCII Ab, anti-mouse CD40 Ab, anti-mouse CD86 Ab, and anti-mouse CD11c Ab were purchased from eBioscience. CCK-8 reagent IL-1β, TNF-α and IL-6 Quantikine ELISA kits were purchased from R&D Systems, Inc. (USA). Ovalbumin (OVA), carbodiimide (EDC), H_2SO_4, HNO_3, N-Hydroxysuccinimide (NHS), Microcystins-LR (MC-LR), keyhole limpet hemocyanin (KLH), fluorescein isothiocyanate (FITC), and lipopolysaccharides (LPS) were purchased from Sigma–Aldrich.

2.2. Mice and cells

6–8 weeks old male BALB/c female mice were obtained from Jiangnan University (Wuxi, China).The mice were maintained under standard conditions according to institutional guidelines and monitored to be pathogen-free. Murine macrophage cell line RAW264.7 was obtained from the Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China), and cultured in a complete DMEM media supplemented with 10% (v/v) FBS, 100 g/mL streptomycin and 100 U/mL penicillin. The DC2.4 cells were from ATCC (American Type Culture Collection), which were cultured in RPMI1640 culture medium supplemented with 10% (v/v) FBS, 100 U/mL penicillin, and 100 g/mL streptomycin. All these cells were grown in a humidified incubator at 37 °C, 5% CO_2.

2.3. Preparation of NP and antigen formulations

Carbon nanotube (MWCNT) was obtained from Nanjing XFNANO Materials TechCo., Ltd. Outer average diameter was between 20 and 30 nm, and length was between 10 and 30 μm. Most studies were carried out using raw CNT, which generally contains microbial contaminants [20]. In order to eliminate the effects of microbial contaminants for the study, we ensured that all experiments were carried out in endotoxin-free conditions using ultra-pure grade reagents during both synthetic, in vitro and in vivo work. Ovalbumin was used as the immunogen.

Ova was conjugated to CNTs in the presence of a water-soluble carbodiimide (EDC) according to the process introduced by Kuiyang Jiang et al. [21], with some modifications. 10 mg CNT was suspended in a 10 mL mixture of 1:3 (V/V) HNO_3 and H_2SO_4, and the SWCNT acid mixture was then subjected to sonication for 6 h. A nanotube mat was obtained after filtration using a 0.05 μm hydrophilized membrane and washed with pure grade water until filtrate became neutral, and then the nanotube was suspended in 15 mL MES buffer solution (50 mM, pH 6.0) by sonication. Then, 10 mL NHS (50 $mgmL^{-1}$) in MES buffer was added to the above suspension. The mixture was sonicated for 30 min followed by addition of 5 mL fresh EDC (15 mgmL−1) in MES buffer. The mixture was stirred for 60 min and then, activated CNT solution was filtered through a 0.05 μm polycarbonate and rinsed thoroughly with MES buffer solution to remove excess EDC, NHS, and byproduct urea. The estered carbon nanotubes were redispersed in 10 mL of MES buffer and then 1.0 mL of a10 mg OVA in MES buffer was added. The reaction was carried out for 6 h. The nanotube suspension was centrifuged and washed with 50 mM MES buffer solution (pH 6.0) to remove unbound protein. The washed protein–nanotube conjugates were dispersed in 50 mM MES buffer solution.

Microcystin-LR (MC-LR), KLH, and MWCNT were also conjugated. In a typical experiment, 2.5 mg of microcystins was dissolved in 0.5 mL of *N*-dimethyl formamide (DMF) followed by addition of freshly prepared EDC solution (3.75 mg of EDC in 0.5 mL DMF) and NHS solution (3.75 mg of NHS in 0.15 mL DMF). The reaction was kept at room temp for 30 min and then kept at 4 °C overnight. The mixture was added slowly to 10 mg KLH which was dissolved in 5 mL of 0.1 M MES buffer. The estered carbon nanotubes were redispersed in 5 mL of MES buffer, and the mixture of KLH and MC-LR were added to estered carbon nanotubes. The reaction was carried out for 6 h. The nanotube suspension was centrifuged and washed with 50 mM MES buffer three times to remove unbound protein.

2.4. Characterization of conjugation

To identify the characterization of OVA-MWCNT, OVA-MWCNT as well as MWCNT and OVA were scanned in the UV–vis spectrum from 200 nm to 400 nm. The protein concentration was measured by BCA method.

The content of the MC-LR was determined by ELISA method to confirm the successful conjugation.

2.5. Cytotoxicity assay

Cells were seeded at 10^4 cells/well (RAW264.7 cell and DC2.4) in 96-well plates. Cell viability was detected using a CCK-8 reagent. Cell were incubated with MWCNT-OVA for 48 h followed by addition of CCK-8 reagent to each well. After incubation at 37 °C and 5% CO_2 for 4 h, the absorbance at 450 nm was measured using a microplate reader.

2.6. In vitro cellular uptake

In vitro studies were carried out using FITC labeled ova in DC2.4 cells. FITC labeled ova were prepared according to Stephanie Konnings et al. [22], with some modifications. First, 5 mg of ova was dissolved in 5 mL of carbonate buffer (0.01 M, pH 9.0). Next, 0.75 mg FITC dissolved in 0.75 mL DMSO was added to the ova solution. The mixture was gently stirred in the dark at 4 °C for 8 h. Then the reaction was terminated by adding 10 μL 0.5 M NH_4Cl. Unbound FITC was removed by dialysis over three days. The resulting FITC-OVA solution was freeze-dried and subsequently stored protected from light at 4 °C.

Flow cytometry technique was used to investigate the effects of particles on antigen uptake, during which DC2.4 cells were seeded