Vaccine 30 (2012) 1560–1571

Contents lists available at SciVerse ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Antigen delivery for cross priming *via* the emulsion vaccine adjuvants

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ARTICLE INFO

Article history:
Received 23 September 2011
Received in revised form
16 December 2011
Accepted 27 December 2011
Available online 9 January 2012

Keywords:
Adjuvants
Antigen delivery
Emulsion
Vaccine

ABSTRACT

The function of emulsion adjuvants in vaccine antigen delivery remains unclear. To investigate the roles of emulsion adjuvants in cross presentation of exogenous antigens, a series of emulsions were prepared for both *in vitro* and *in vivo* studies. Bone marrow-derived dendritic cells (BMDCs) were treated with the adjuvants and analyzed by flow cytometry for the expression of costimulatory molecules. The activation of antigen-specific T cells *in vitro* was determined with B3Z cells. Antibody secretion in the draining lymph nodes of emulsion adjuvant-treated animals was measured by enzyme-linked immuno-spot (ELISPOT) assays, and antigen-specific proliferation of cells was conducted to examine the roles of emulsion adjuvants in antigen delivery. Data on phagocytosis of adjuvant-treated cells correlated well with the degree of cell death induced by the emulsion adjuvants. Significant inflammatory infiltration and cell death were observed *in vivo* at the adjuvant injection sites, as demonstrated by hematoxylin and eosin (H&E) staining and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays. Ovalbumin (OVA)-based ELISPOT assays showed that L121-adjuvant, containing Pluronic L121, induced the most significant cell death also stimulated the strongest antibody-producing response in the draining lymph nodes, consistent with the data on the proliferation of antigen-specific T cells and activation of B3Z cells *in vitro*. Results presented in this study have demonstrated the roles of emulsion adjuvants in induction of cell death and delivery of exogenous antigens for cross-priming, leading to stimulation of antigen-specific immune responses.

1. Introduction

Emulsion vaccine adjuvants have been widely used to enhance immune responses to co-administered antigens. Despite the extensive use of emulsion adjuvants for the activation of adaptive immunity, the underlying mechanism by which these adjuvants stimulate antigen-specific responses remains unclear. One hypothesis is that the vaccine adjuvants serve as an antigen depot at the local injection site and, upon vaccination, professional antigen presenting cells (APCs) take up the antigens and process and present them to T cells *via* the major histocompatibility complex (MHC) to induce the maturation and triggering of T cell activation. Based on our previous studies using a series of emulsion adjuvants, we have shown that the dispersion properties and antigen release rate of the vaccine delivery system may not be directly related to the dispersion properties of the adjuvants [1] and that treating APCs with emulsion adjuvants in the presence of adjuvant-induced dead cells enhanced antigen delivery *via* phagocytosis and macropinocytosis [2]. It was hypothesized that the extent of cross presentation of antigens may correlate with the degree of cell death induced by the adjuvants. To test this hypothesis, the effect of vaccine adjuvants on cross-presentation of exogenous antigens was examined in this study based on the emulsion system established previously [1]. To investigate the relationship between immunogenicity and the physicochemical properties of the emulsion adjuvants, a number of emulsion adjuvants containing surfactants with various hydrophile–lipophile balance (HLB) values were prepared, as previously described [1], and the ability of these compounds to stimulate antigen-specific immune responses *in vivo* was examined. Alum, an aluminum-containing adjuvant used in humans [3], was included in this study for comparison purposes.

Dendritic cells are the most potent antigen-presenting cells for triggering an adaptive immune response. These professional APCs are able to acquire exogenous antigens from dying cells and present them to MHC class I-restricted $CD8^+$ cytotoxic lymphocytes (CTLs) [4–6]. The role of emulsion adjuvants has been revealed in our previous studies, showing that surfactant-containing vaccine adjuvants induced cell death, including both apoptosis and necrosis, which result in cross-priming with exogenous antigens [7–9]. The activation of DCs by danger signals is known to induce local

Abbreviations: Adj., adjuvant; APCs, antigen-presenting cells; BMDCs, bone marrow-derived dendritic cells; CFSE, carboxyfluorescein succinimidyl ester; CTL, cytotoxic lymphocyte; ELISPOT, enzyme-linked immunospot; FDG, fluorescein di-β-D-galactopyranoside; GM-CSF, granulocyte–macrophage colony-stimulating factor; HBS, HEPES-buffered saline; HLB, hydrophile–lipophile balance; HRP, horse radish peroxidase; L121, Pluoronic L121; MHC, major histocompatibility complex; OVA, ovalbumin; PBS, phosphate-buffered saline; Sp, Span; Tw, Tween.

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doi:10.1016/j.vaccine.2011.12.120

inflammation and DC maturation, characterized by the enhanced expression of costimulatory molecules and MHC complexes. To examine the physicochemical properties of the emulsion adjuvants and their ability to induce cell death and trigger dendritic cells to engulf antigen-bearing dying cells *in vitro*, we cocultured bone marrow-derived dendritic cells (BMDCs) with adjuvant-treated cells and then examined the degree of engulfment and the expression of costimulatory molecules, including CD40, CD80 and CD86, on the BMDCs. Cell death induced by the emulsion adjuvants *in vivo* was examined by histochemical staining of the subcutaneous tissues at the injection site after immunization and detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays. To investigate the ovalbumin (OVA)-specific immune response induced by the vaccine adjuvants, cells in the lymph nodes were isolated after immunization and examined by enzyme-linked immunospot (ELISPOT) assays to quantify the numbers of antibody-secreting cells. Antigen-specific proliferation of T cells in the draining lymph nodes was assayed by labeling the cells in the lymph nodes with methyl-[^{3}H] thymidine and measuring their subsequent radioactivity with a β-counter; and the kinetics of antigen delivery *via* the vaccine adjuvants was also examined by flow cytometry. Stimulation of OVA-specific antigen presentation, on the other hand, was assessed by the *in vitro* stimulation of B3Z cells, a V_β5-expressing cytotoxic T-cell clone that specifically recognizes the OVA/Kb ligand [10].

The results of this study showed that emulsion adjuvant-induced dead cells were phagocytosed by dendritic cells, resulting in the upregulation of costimulatory molecules, including CD40, CD80, CD86, and MHC II. The emulsion adjuvants induced T cell proliferation and stimulated OVA-specific B cells in the draining lymph nodes. However, most emulsion adjuvants, except L121-adjuvant, did not induce significant B3Z T cell activation. Kinetic studies demonstrated that L121-adjuvant was the most efficient at stimulating the delivery of soluble antigens to the cells in the lymph nodes. The ability of the emulsion adjuvants to facilitate antigen delivery is clearly demonstrated in this study.

2. Materials and methods

2.1. Materials

Aluminum hydroxide gel adjuvant (Alhydrogel 2.0%) was purchased from Brenntag Biosector (Frederikssund, Denmark). The antibodies used in this study, including anti-CD40 (HM40-3), anti-CD80 (16-10A1), anti-CD86 (GL1), anti-MHC-II (M5/114.15.2), anti-CD11c (N418) and the isotype controls, were obtained from eBioscience Inc. (San Diego, CA).

2.2. Preparation of the emulsion adjuvants

The emulsion adjuvants were prepared by ultrasonication as described previously [1]. Briefly, dispersions of the non-miscible phases of squalane and phosphate-buffered saline (PBS) were emulsified at 50:50 (w/w) with 2% emulsifier with varying hydrophile–lipophile balance (HLB) values, including Span 85, Span 20, Tween 85, Tween 80, and Tween 20. The emulsifiers in the Span (20 + 85)-adjuvant were composed of a 30:70 blend of Span 85 and Span 20. These preparations were formulated with identical compositions except the emulsifiers, so that the effect of dispersion properties, either w/o- or o/w-types, on antigen delivery and presentation can be compared. L121-adjuvant, on the other hand, was composed of 3.75% Pluronic L121, 0.6% (w/w) Tween 80, and 15% (w/w) squalane [11]. Adjuvant preparations containing Spans, such as Span 20, Span 85, and Tween 85, are w/o-type emulsions, while the rest are o/w-type emulsions.

2.3. Cells

EL4 is an H-2b thymoma cell line, which was maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 4 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS). EG7-OVA is a transfected EL-4 cell line expressing the full-length ovalbumin cDNA that was maintained in DMEM containing 400 μg/ml G418 [12]. B3Z is a hybridoma cell line generated by the fusion of a Vβ5-expressing CTL clone specific for H-2 K^b/OVA and a cell line expressing a nuclear factor of activated T cells (NFAT)-driven *lacZ* gene [13,14]. This cell line was used to measure specific H-2 K^b/OVA responses.

Dendritic cells were isolated from the bone marrow of C57BL/6J mice using the established procedures [15]. Briefly, bone marrow cells were extruded from the femurs and tibias of the animals. After the lysis of red blood cells with ammonium chloride–potassium (ACK) buffer, the cells were maintained in 24-well plates for 6 days in RPMI 1640 medium containing β-mercaptoethanol, mGM-CSF, and mIL-4. The cells were gently washed on days 2 and 4 to remove floating cells, and fresh medium containing GM-CSF and IL-4 was added. On day 6, the cultures were transferred to fresh wells containing culture medium with mGM-CSF and mIL-4 for use in experiments. The animals care was approved and performed in accordance with our institutional guidelines.

2.4. Phagocytosis of adjuvant-induced apoptotic and necrotic cells by BMDCs

To determine the degree of uptake of adjuvant-induced apoptotic and necrotic cells by BMDCs, EL4 cells were labeled with PKH-67 (Sigma), washed with PBS, treated with 300 μl of emulsion adjuvant for 15 min, washed again with PBS, and incubated at 37 °C for 24 h. BMDCs were then cocultured at 1:1 with the adjuvant-treated EL4 cells at 37 °C for 6 h and washed with PBS to remove floating cells. Cells were then stained with PE-conjugated anti-CD11c antibody and analyzed using a FACSCalibur immunocytometry system (Becton-Dickson, Immunocytometry System, San Jose, CA).

2.5. Activation of costimulatory molecules in BMDCs by adjuvant-treated EL4 cells

To examine the effect of the emulsion adjuvants on maturation of dendritic cells, EL4 cells were pre-treated with 300 μl emulsion adjuvant for 15 min, washed with PBS, and incubated at 37 °C for 24 h. Day 6 BMDCs were cocultured with adjuvant-treated EL4 cells for 48 h, washed with PBS, and stained with antibodies against MHC class II, CD40, CD80, and CD86 for 30 min at 4 °C, followed by flow cytometric analysis of CD11c+ cells using a FACSCalibur.

2.6. Hematoxylin and eosin (H&E) staining and in situ detection of apoptosis by TUNEL

To examine inflammatory infiltration and cell death after treatment with the emulsion adjuvants, the injection sites were excised 24 h after vaccination, sectioned, and stained with hematoxylin and eosin (H&E) for microscopic examination. Cell death at the vaccination sites was examined by TUNEL assay using the terminal deoxynucleotidyltransferase (TdT)-FrgEL^{TM} DNA fragmentation detection kit (Oncogene Research Products) according to the manufacturer's instructions. Briefly, after deparaffinization and rehydration, 5-μm paraffin-embedded tissue sections were incubated at 37 °C with proteinase K (20 μg/ml) for 20 min and rinsed with Tris-buffered saline (TBS; 20 mM Tris, pH 7.6, and