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Application of pH-sensitive fusogenic polymer-modified liposomes for development of mucosal vaccines

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ABSTRACT

To evaluate the usefulness of pH-sensitive fusogenic polymer (succinylated poly(glycidol) (SucPG) and 3-methylglutarylated poly(glycidol) (MGluPG))-modified liposomes as mucosal vaccine in the induction of a protective immune responses was evaluated. Mice were nasally immunized with OVA-containing SucPG-modified liposomes. After immunization, significant Ag-specific Abs were detected in the serum and intestine. When sera were analyzed for isotype distribution, antigen-specific IgG1 Ab responses were noted in mice immunized with OVA-containing polymer-unmodified liposomes, whereas immunization with OVA-containing SucPG-modified liposomes resulted in the induction of OVA-specific IgG1, IgG2a and IgG3 Ab responses. In spleen lymphocytes from mice immunized with OVA-containing SucPG-modified liposomes, both IFN-γ and IL-4 mRNA were detected. The same result was obtained also in the mouse immunized with OVA-containing MGluPG-modified liposomes. Furthermore, we examined the induction of immune responses in chickens following intraocular immunization with *Salmonella* Enteritidis Ag-containing MGluPG-modified liposomes, and the protective effect against the challenge with *S.* Enteritidis. Immunization with *S.* Enteritidis Ag-containing MGluPG-modified liposomes induced significant Ab responses against *S.* Enteritidis in the serum and intestine. Less fecal excretion of bacteria was observed in chickens immunized with *S.* Enteritidis Ag-containing MGluPG-modified liposomes after challenge. The numbers of bacteria in the caecum were also lower in immunized chickens than in unimmunized controls.

1. Introduction

Many pathogens cause disease by first colonizing or penetrating through the mucosal surface of the enteric epithelium (Beachey, 1981; Edwards and Puente, 1998; Khan et al., 2000; Klemm and Schembri, 2000). The mucosal immune system plays a central role in the primary defense against pathogens by preventing binding of the microbes or their toxins to the epithelium (Williams and Gibbons, 1972; Winner et al., 1991; Cotter et al., 1995). Externally secreted IgA and local IgG Abs produced in response to the mucosal invasion or administration of antigens perform important functions in this system (Bouvet and Fischetti, 1999). It has been reported that these local Abs are effective in inhibiting the binding of pathogen to the mucosal cells (Bouvet and Fischetti, 1999). Therefore, an effective strategy for the clearance of pathogens from sites of invasion, such as aero-digestive and reproductive tract mucosa, would

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be to induce the local immunity against pathogens in the invasion sites in addition to systemic immune responses following immunization.

Liposomes are lipid multilayer vesicles that have been successfully used as delivery systems for antigens, drugs and genomic material (Gregoriadis, 1990). Use of liposome-associated Ags is known to induce protective immunity against microbial infections (Gregory et al., 1986; Kahl et al., 1990; Bulow and Boothroyd, 1991; Li et al., 2004; Irie et al., 2005). To establish effective vaccine, moreover, we have developed pH-sensitive liposomes, which generate fusion ability under weakly acidic conditions, by surface modification of liposomes with pH-sensitive fusogenic polymer having carboxyl groups, such as succinylated poly(glycidol) (SucPG) and 3-methylglutarylated poly(glycidol) (MGluPG) (Yuba et al., 2010). However, relatively little data on their potential mucosal vaccine is inconclusive.

Salmonellosis is one of the most important intestinal infectious diseases in the poultry industry (Hopper and Mawer, 1988; Cooper et al., 1989) Many attempts have been made to control *Salmonella* infections in chickens following epidemiological analyses of human salmonellosis caused by *Salmonella enterica* serovar Enteritidis that showed contaminated chicken eggs or egg products are major sources of the infection (St. Louis et al., 1988; Hansenson et al., 1992). Various killed *Salmonella* vaccines (Barbour et al., 1993; Gast et al., 1993; Nakamura et al., 1994; Miyamoto et al., 1999) and attenuated vaccines (Barrow et al., 1991; Cooper et al., 1994; Hassan and Curtiss, 1997; Allen et al., 2000) have been developed and reported as effective in reducing the shedding of *Salmonella* in feces and the dissemination of the bacteria to internal organs. Mucosal surfaces are the major place of entry of *Salmonella* into the body. It is therefore crucial to induce a protective immune response in the intestinal mucosa of chickens in order to develop an immunological control strategy.

To know the usefulness of pH-sensitive fusogenic polymer-modified liposomes as mucosal vaccine, OVA-containing SucPG-modified liposomes were nasally inoculated to mice and immune responses were evaluated. In addition, a possibility of the control of *Salmonella* infections in chickens following intraocular immunization with *S.* Enteritidis Ag-containing MGluPG-modified liposomes was examined.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylethanolamine (DOPE), monophosphoryl lipid A (MPL), trypsin inhibitor (soybean type I-S), and OVA (SIGMA) were commercial products. SucPG and MGluPG were prepared as previously reported (Sakaguchi et al., 2008; Kono et al., 1994). Molar percentages of glycidol/carboxylated glycidol/n-decylamine-attached units in the resultant SucPG or MGluPG polymer were determined by ^{1}H NMR to be 18/74/8, 9/89/11 respectively (Yuba et al., 2010).

2.2. Animals

Female BALB/c mice (6 weeks old) were purchased from Charles River Japan, Tokyo, Japan. Three-week-old SPF Hy-Line strain chicks were obtained from the Nippon Institute for Biological Science, Ohme, Tokyo, Japan, and were maintained in a *Salmonella*-free environment. All animals were maintained according to the Standards Relating to the Care and Management of Experimental Animals of Japan. The experiments were carried out in accordance with the guidelines for animal experimentation in Osaka Prefecture University.

2.3. Bacteria and preparation of bacterial Ag

S. Enteritidis strain 1227 was kindly provided by Dr. Y. Adachi (School of Agriculture, Ibaraki University, Ibaraki, Japan). The organism was originally isolated from a chick affected with the bacteria. *S.* Enteritidis Ag was prepared as follows. The bacteria were cultivated in Tryptosoya broth (Nissui Pharmaceutical) for 6 h at 37 °C with continuous shaking. Formaldehyde solution was then added up to a concentration of 1%. The suspension was gently shaken overnight to deactivate the bacteria. The formalin was removed by centrifuging the cells three times with PBS (pH 7.4). Cell lysate of the bacteria was then prepared by ultrasound irradiation of a 10% bacterial cell suspension for 15 min three times (BRANSON Sonifier 250, Emerson Japan). Death of the bacteria was confirmed by absence of growth when 100 μl of the suspension was inoculated onto Heart Infusion agar (Nissui Pharmaceutical Co.).

2.4. Preparation of liposomes

Polymer (SucPG or MGluPG)-modified liposomes that entrap OVA or *S.* Enteritidis Ag were prepared by the following method. DPPC (4 μmol), DOPE (4 μmol), MPL (16 μg), and SucPG or MGluPG polymer (lipids/polymer = 7/3, w/w), each dissolved in an organic solvent (DPPC and DOPE, chloroform–methanol = 2:1, v/v; MPL, chloroform–methanol = 1:2, v/v; SucPG and MGluPG polymer, methanol), were mixed in a conical flask. The lipids were dried on a rotary evaporator, and left to stand for 30 min in a high vacuum in a desiccator. After addition of 1 ml of PBS containing OVA (5 mg/ml) or *S.* Enteritidis Ag (5 mg/ml) and incubation at an appropriate temperature for 3 min, the lipid film was dispersed by vigorous vortexing. Any unencapsulated OVA or *S.* Enteritidis Ag was removed by repeated centrifuging at 14,000 × *g* for 20 min at 4 °C in PBS, and the resulting liposome suspension was used for immunization.

Polymer-unmodified liposomes that entrap OVA were prepared from lipid mixture solution containing DPPC (4 μmol), DOPE (4 μmol), and MPL (16 μg) as stated above.

The amount of OVA or *S.* Enteritidis antigen entrapped in liposomes was determined by the following method. Ninety microliters of isopropyl alcohol was added to a 10 μl suspension of liposome-entrapped OVA or *S.* Enteritidis Ag (at three-fold dilution in PBS), followed by vortex mixing. The protein concentration of the resulting solutions was determined using a Bio-Rad protein assay kit (Bio-Rad