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Ginsenoside Rd induces protective anti-*Candida albicans* antibody through immunological adjuvant activity

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ABSTRACT

The role of an antibody against candidiasis is controversial. However, a certain *Candida albicans* surface epitope produces a protective antibody. Yet, its isolation is difficult. In this study, we investigated if ginsenoside Rd from *Panax ginseng* has an immunoadjuvant ability to induce surface mannan extract (CASM) to produce a protective antibody. Mice were immunized twice i.p. with an emulsion form of CASM mixed with one of the following: IFA [CASM/IFA], or CFA [CASM/CFA] or Rd with IFA [CASM/Rd/IFA]. One week after the booster, these mice were challenged i.v. with live *C. albicans* and their survivability was measured. Results showed that four of five CASM/Rd/IFA-vaccinated mice survived during the entire 110 day-observation period, whereas CASM/IFA- or CASM/CFA-vaccinated mice died within 19 and 23 days ($P < 0.05$). The antiserum from CASM/Rd/IFA-immunized mice transferred the protection to naïve mice, whereas antiserum from CASM/CFA-given mice was not protective although CASM/CFA induced an antibody four times greater than CASM/Rd/IFA. IgG isotyping revealed that CASM/Rd/IFA-vaccine produced the most abundant IgG and IgG2a—resulting in the highest ratio (1.32) of IgG2a to IgG, which is helpful in treating Th2-oriented candidiasis. In contrast, the formulae lacking Rd had these ratios less than 1. This strongly indicates that Rd could enhance Th1 immunity. Cytokine profiles and DTH further confirmed the Th1 dominance. Rd caused no hemolysis. Combining all of these data together, Rd can enhance Th1-response to CASM in mice. This protects mice against disseminated candidiasis by eliciting higher titers of Th1 type antibody and a Th1-dominant immune response.

1. Introduction

One of the major challenges in vaccine development is ensuring a proper response during the induction of a particular pathogen because the wrong response may further aggravate the infectious disease. *Candida albicans* is one of those pathogens. In other words, generally, antibodies have no protective ability against fungal infections. Thus, the role of antibodies against *C. albicans* infection has been controversial. Some researchers have shown that antibodies do not protect mice against experimental disease [1,2], and others have reported that patients still develop hematogenously disseminated candidiasis despite having anti-candidal antibodies in their serum [3,4]. However, we [5] and others [6–14] have found that a certain cell surface epitope from the fungus induces protective effects. For example, monoclonal antibody (mAb) B6.1 is protective, whereas mAb B6 has no protective ability even though the epitopes of the two mAbs are both located on the cell surface of *C. albicans* [15–17]. These findings indicate that the important issue in antibody development is about selecting a "good" antigenic source that induces protective antibodies. Unfortunately, the isolation of such an antigenic source is fastidious and time-consuming—not to mention, expensive. Additionally, the formation of a liposomal vaccine is complicated and requires more than five immunizations [5]. Although it only requires two immunizations, conjugate vaccine formulation is even more complicated [18]. This led us to discover a simple and inexpensive way of developing fungal vaccines against *C. albicans* infections.

In the immunoregulation of the CD4+ T helper (Th) cell, Type 1 (Th1) and Type 2 (Th2) immune responses demonstrate essentially different and opposite effector functions [19,20]. A Th2-involvement can be deleterious to the patient while Th1 immunity would be protective and vice versa. In *C. albicans* infections, the dominant immune response is correlated to the severity of the fungal infection with Th1-dominance reducing the severity [21,22]. Th1 and Th2 immunities can be distinguished by the cytokine and IgG isotype profiles. The Th1 immunity, which is a requisite for cytotoxic T-lymphocyte (CTL) production, is characterized by dominant productions of IFN-γ and IL-2 and of IgG2a in mice, whereas Th2 is characterized by the production of IL-4 and IL-10 and enhancement of IgG1 induction. Adjuvants can affect the immune response and cause the immune system to tilt in the favor of either the Th1 or Th2

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immune response [20]. Thus, any adjuvant that induces a Th1 dominant immunity would be advantageous for the formulation of a fungal vaccine. Currently, one of the only adjuvants used in humans, alum, elicits Th2 dominant immunity [20,23,24]. In regard to fungal adjuvants, prospects for development of vaccines have been determined [25–27], but still these fungal adjuvants appear to be at experimental levels.

Ginsenosides are mostly known as being responsible for immunological activities [28,29]. Of the ten different types of ginsenosides isolated from *Panax ginseng*, only ginsenoside Rd, which belongs to the protopanaxdiol family, was helpful in the production of antibody by *C. albicans* carbohydrate antigen as analyzed by adjuvanticity analysis [30]. In that study, the protective ability of the antibody and the type of immunity were not determined. In addition to our reports about Rd, Yang and his co-workers [31] reported that Rd had immunological adjuvant activity which elicited both Th1 and Th2 immunities in the protein ovalbumin (OVA)-immunized mice. Besides these reports, little information about Rd's immunological adjuvant activity was found. Therefore, the ability of Rd to induce a *C. albicans* surface mannan protective antibody against experimental disseminated candidiasis was examined. Additionally, the possible immunological mechanism of the protection was investigated.

2. Materials and methods

2.1. Organisms and culture conditions

C. albicans strains of 1 and A9, which have been previously characterized [5,32,33], were grown in glucose-yeast extract-peptone (GYEP) broth at 37 °C as described previously [5,32,33]. To challenge the mice, the yeast form of *C. albicans* was prepared by collecting yeast cells from broth culture, washing the cells with cold sterile Dulbecco's phosphate-buffered saline solution (DPBS; Sigma, St. Louis, USA), and enumerating with the use of a hemocytometer to obtain the desired numbers of yeast cells.

2.2. Mice

Female BALB/c mice at 6 weeks of age were purchased from Charles River Lab, USA and used in experiments. These mice were maintained in the animal facility under the Dongduk Women's University's regulation.

2.3. Ginsenoside Rd

Ginsenoside Rd with a purity of 98% was isolated from the root of *P. ginseng* by following the procedures as previously described [34]. Prior to use in the experiments, the ginsenoside Rd was tested for the presence of endotoxins by the Limulus amebocyte lysate test (E-Toxate Kit; Sigma) by following the manufacturer's guidelines. Rd was dissolved in sterile DPBS at a desired concentration and filter-sterilized (a pore size = 0.2 μm; Sartorius, Goettingen, Germany) before use. The filtered Rd was inoculated on a blood agar plate (Korean Culture Media, Seoul, Korea) in order to ensure that there was no microbial contamination. The filtered Rd had no microbial growth or endotoxin content under the conditions of the commercial kit.

2.4. Anti-candidal activity of ginsenoside Rd, in vitro

To determine whether or not Rd alone has a growth-inhibitory activity against *C. albicans*, an agar-diffusion susceptibility method was utilized as previously described [22,24]. In brief, *C. albicans* (5×10^6 yeast cells/ml) was inoculated with sterile swabs on a Mycobiotic agar (Difco, Sparks, MA) plate, wells were made on the plates with a metallic puncher, and 100 μl of Rd in DPBS (Sigma, St. Louis, MO, USA) at various concentrations from 0 to 100 μg/ml was put into the designated wells, respectively. A negative control well received the same volume of only diluent (DPBS). As a positive control, amphotericin B was used instead of Rd. All plates were incubated at37°C for 48 h, and growth-inhibitory zones were measured.

2.5. Vaccine formulations

Ginsenoside Rd (1 mg/ml) with the yeast cell surface mannan extract (CASM; 400 μg) was dissolved in sterile Dulbecco's phosphate buffered saline (DPBS; Sigma, St. Louis, MO, USA) and mixed with Incomplete Freund's Adjuvant (Difco, Detroit, MI, USA) [CASM/Rd/IFA] at a 1:1 ratio by volume. As a control formula, Rd addition was omitted from the [CASM/Rd/IFA], and this formula was referred as [CASM/IFA]. As another control formulation, a mixture of CASM plus Complete Freund's Adjuvant (CFA; Difco) [CASM/CFA] was used instead of IFA. All of the mixtures were emulsified by sonification (Sonic & Materials, Inc., Newtown, CT, USA). The CASM (2-ME extract or mannan extract) was obtained by β-mercaptoethanol extraction as previously described [5,33,35,36]. Chemical analysis showed that the CASM primarily consisted of mannan with approximately 3.5% protein [5].

2.6. Protective effect of active immunization

Mice were divided into three groups of five. Each group was intraperitoneally (i.p.) immunized twice with either CASM/Rd/IFA or CASM/IFA or CASM/CFA formula, respectively, during a 21 day interval. The volume of one vaccination was 200 μl per mouse. One week after the booster, the animals were challenged with live *C. albicans* (5×10^5 per mouse in 0.2 ml volume) via intravenous (i.v.) route [5,17,18]. Another control mice group received only diluent (DPBS) before an i.v. challenge. Relative resistance or susceptibility to disseminated candidiasis was assessed by measuring survival times.

2.7. Passive immunization test

To determine the ability of the antibody to transfer resistance against disseminated candidiasis to naïve mice, the antiserum that was obtained from [CASM/Rd/IFA]-vaccinated mice was tested. Experiments for the test were done by the procedure as described previously [5]. In brief, mice were given 0.5 ml of the antiserum via i.p. route, and 4 hours later, the animals were challenged with live *C. albicans* yeast cells (5×10^5/mouse in 0.2 ml volume) i.v. 48 hours after the challenge; the numbers of colony forming units (CFU) per gram of kidneys in the animals were determined as described previously [5]. The kidney is known to be an indicator of severity in disseminated candidiasis [37,38]. As controls, normal mouse serum (NMS), antiserum heated for 30 min at 56 °C before use, and antiserum adsorbed by dead *C. albicans* cells were also tested in the same manner as intact antiserum from [CASM/Rd/IFA]-immunized mice.

2.8. Detection, titration, and IgG isotyping of antibodies specific for CASM in the antisera

To detect antibody presence, polyclonal antisera were collected from mice that were immunized in the same way as described above. One week after the booster, in place of the challenge, the sera were collected. The determination was done by an agglutinin assay against CASM-coated latex beads as described before [5,16]. In brief, a constant amount of the latex beads (10 μl) was mixed with an equal volume of two fold serially diluted antiserum in DPBS on O-ring slides. Then, a reciprocal number of the greatest dilution that formed agglutination was evaluated as the antibody titer. The IgG isotyping analysis in the antisera was measured by ELISA as describe previously [39]. The antisera were diluted 1:50 in DPBS.