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Comparison of Two *Candida* Mannan Vaccines: The Role of Complement in Protection against Disseminated Candidiasis

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We have previously shown that *Candida albicans* mannan extract encapsulated in liposomes [Lipo-mann] or conjugated to a protein (bovine serum albumin) [Conju-mann] induces the production of antibody in BALB/c mice with normal complement system that protect against disseminated candidiasis. In this present study, we determined the protective abilities of two formulae in a C5-deficient mouse model of disseminated candidiasis. It is known that the lack of C5 is known to aggravate candidal infection. In experiments, BALB/c or C5-deficient mice-DBA/2J and AKR mice, were immunized with one of the formulae before intravenous challenge with live *C. albicans* yeast cells and their degrees of survivability were measured. Results showed that Conju-mann was 100% protective in BALB/c mice against disseminated candidiasis, whereas only 60% of Lipo-mann immunized mice survived the entire 50 day observation period ($p < 0.05$). With the DBA/2J strain, Conju-mann resulted in a partial protection, but Lipo-mann had no protection. The conjugate vaccine enhanced the resistance of AKR mice, which resulted in three survivors of the five Conju-immunized AKR mice until the end of 50 day observation period ($p < 0.05$). Lipo-mann showed little protection in AKR mice. By agglutination analyses, it was determined that there was the same level of production of polyclonal antisera specific to the mannan regardless of the mouse strains. All data indicate that both formulations require complement in the protection. However, Conju-mann appears to be superior to Lipo-mann because the conjugate vaccine is protective even in the absence of C5. These observations suggest that the conjugate vaccine can be an excellent vaccine formulation against *C. alibicans* infections.

Key words: *Candida albicans*, Mannan-vaccines, C5-deficient, Disseminated candidiasis, Complement, Antibody titer

INTRODUCTION

Previously, we found that the cell wall mannan of *Candida albicans* is responsible for the binding of *C. albicans* yeast cells to mouse splenic marginal zone macrophages (Kanbe et al., 1993) and to subcapsular sinus areas of peripheral lymph nodes (Han et al., 1993). With the use of this mannan extract as an antigenic source, two types of vaccines were developed. The first type of vaccine was formulated by encapsulating the mannan into liposomes (Lipo-mann) that consisted of phosphatidyl choline and cholesterol (Han and Cutler, 1995). In an animal model of hematogeneously disseminated candidiasis, the Lipo-mann vaccine enhances resistance of the BALB/c strain of an inbred mouse when challenged intravenously (i.v.) with a lethal dose of live *C. albicans* (Han and Cutler, 1995). The protection is partly due to the antibody. The second type of vaccine was later developed by conjugating the mannan (Conju-mann) to bovine serum (BSA) as a protein carrier (Han et al., 1999).

The polyclonal antiserum obtained from the Conju-mann-immunized BALB/c mice has the same ability to transfer protection to naïve mice as the antiserum obtained from the Lipo-mann-immunized BALB/c mice does (Han and Cutler, 1995; Han et al., 1999). When comparing the two formulae, it seems that Conju-mann

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is more convenient than Lipo-mann because Conju-mann protects mice by a priming dose followed by a single booster (Han et al., 1999). In contrast, Lipo-mann requires more than five immunizations for a proper antibody induction to occur (Han and Cutler, 1995).

According to others' reports, the degree of severity by *C. albicans* infection varies and is dependent on the type of mouse strain. That is, the BALB/c strain is found to be the most resistant to systemic infection, whereas DBA/2J strain is the most susceptible (Costantino et al., 1995; Ashman et al., 1996, 2003). And, the susceptibility of the AKR strain is known to be moderate (Ashman et al., 1996). These reports are not surprising because DBA/2J and AKR strains are known to have an abnormal complement system in their bodies and are both C5-deficient (Ashman et al., 1996). The C5 is one of the major, common components that play an important role. The lack of the component aggravates candidal infections (Mullick et al., 2006; Radovanovic et al., 2011; Cheng et al., 2012). Our previous work shows that the third component of complement (C3) is involved in the protection against disseminated candidiasis through monoclonal antibody (MAb) B6.1 specific to the mannan (Han et al., 2001). MAb B6.1 isolated from Lipo-mann-immunized BALB/c mice is protective against disseminated candidiasis and vaginal infection (Caesar-TonThat and Cutler, 1997; Han et al., 1997, 1998, 2000). Although this finding indicates that complement is essential in that protection, there is no direct evidence that complement is involved in protection provoked by active immunization provided by the two *Candida* vaccines mentioned above. In this study, we investigated the role of complement in the protection provoked by the two vaccines. In addition, we further examined both vaccines to see which formulation has more potential in the protection against disseminated candidiasis. Thus, the effectiveness of Lipo-mann and Conju-mann formulations were compared by using a C5-deficient mouse model of disseminated candidiasis.

MATERIALS AND METHODS

Organism and culture conditions

C. albicans strain 1 (CA-1) that was previously characterized (Hazen et al., 1991; Kanbe et al., 1993) and used in our other previous work (Han, 2005, 2010; Lee and Han, 2006; Lee et al., 2008; Kim et al., 2012) was grown in GYEP (glucose-yeast extract-peptone) broth as previously described (Han and Cutler, 1995; Han et al., 2001). The yeast form of the *C. albicans* was washed with cold sterile DPBS (Dulbecco's phosphate saline solution; Sigma) and then was used for an intravenous challenge to measure survival rates in an animal model against disseminated candidiasis.

Mice

6 to 7 week old, female ALB/c, AKR, and DBA/2J (Jackson Laboratory) mice were used. The animals were maintained in the animal facilities under pathogen-free conditions in accordance with the guidelines of the institutional animal care of Dongduk Women's University.

Formulations of mannan vaccines

The Lipo-mann was prepared as previously described (Han and Cutler, 1995). In brief, mannan was extracted from *C. albicans* cell wall and was encapsulated into multilamellar liposomes that consisted of phosphatidylcholine and cholesterol at a molar ratio of phosphatidylcholine to cholesterol of approximately 3.2:1. The amount of mannan extract within the Lipo-mann vaccine was 178 g/mL, which was determined by the phenol-sulfuric acid reaction (Han and Cutler, 1995). Control liposomes were prepared exactly as described above, but a buffer (DPBS) without mannan extract was added. Preparation of Conju-mann was done by conjugation of CNBr-activated mannan to BSA in presence of 1-ethyl-3 (3-dimethylaminoprophyl) carbodiamide-HCl (EDC; Sigma) (Han et al., 1999). The mannan was obtained in crude form as previously done before (Kanbe et al., 1993; Kanbe and Cutler, 1994; Han and Cutler 1995) by a 2-mercaptoethanol extraction of the CA-1 strain.

Evaluation of the vaccines

Test mice were immunized, intravenously (i.v.), with the Lipo-mann vaccine for five times on a weekly basis as previously described (Han and Cutler, 1995; Han and Lee, 2005). One week after the last booster, the mice were challenged with live *C. albicans* (5×10^5 per mouse in 0.2 mL volume) (Han and Cutler, 1995; Han et al., 2001). Control mice groups received liposome only (Lipo-DPBS) or diluent (DPBS) only before an i.v. challenge. In the case of the Conju-mann formula, mice were intraperitoneally (i.p) immunized with formula mixed with an equal volume of adjuvant (R-700; RIBI ImmunoChem Research) twice in a 21 day interval. Then, one week after the booster, the animals were challenged with live *C. albicans* at the same dose and by the same route as the Lipo-mann-immunized mice as described before (Han et al., 1999). The effects of these vaccines on the host defense of mice against the disseminated candidiasis were evaluated by comparing mean survival times.

Antibody titer

To determine if complement deficiency causes an induction of antibody, polyclonal antiserum samples were obtained from mice that were immunized with Lipo-mann or Conju-mann as described above for evaluation of the vaccine. Measurement of the antibody inductions was done by an agglutination assay against mannan-coated latex beads as described before (Han et al., 1998). In brief, a constant amount of the latex beads (10 μL) was mixed with a same volume of each of the serial two fold dilutions of an antiserum sample in DPBS on a O-ring (diameter = 10 mm) glass plate. Then the greatest dilution that formed agglutinin was determined while gently stirring the plate. The reciprocal number of the factor of the greatest dilution was evaluated as an antibody titer of the test antiserum sample.

Statistics

Statistical significance of differences in survival times was calculated by the Kaplan-Meier test computed with Systat 7.0 (New Statistics for windows; SPSS). In other analyses, statistical significance of the difference between test and control was determined by Student's *t* test.

RESULTS

The prophylaxis of Lipo-mann and Conju-mann against disseminated candidiasis

During the entire 50 day observation period, the mean survival times (± S.E.) showed that the BALB/c mice immunized with Lipo-mann survived an average of 17 days longer than the control BALB/c mice groups given DPBS or Lipo-DPBS ($p < 0.05$) (Fig. 1A). Three of the five immunized mice survived the entire observation period. When the BALB/c mice were immunized with Conju-mann, all five mice survived the entire period; whereas control BALB/c mice groups given adjuvant or DPBS all died within 15 and 18 days, respectively (Fig. 1B). When the degrees of survival rates were compared to each other, the Conju-mann formula provoked a survival rate greater than that of the Lipo-mann formula (Fig. 1).

Prophylactic effects of Lipo-mann and Conju-mann on DBA/2J mice against disseminated candidiasis

Lipo-mann-immunized DBA/2J mice groups had a survival value of (8.4 ± 1.8) days (Fig. 2A). This survival value was not very different from those of the negative control mice groups resulting in (6.4 ± 1.5) days and (6.2 ± 0.5) days corresponding to DPBS and

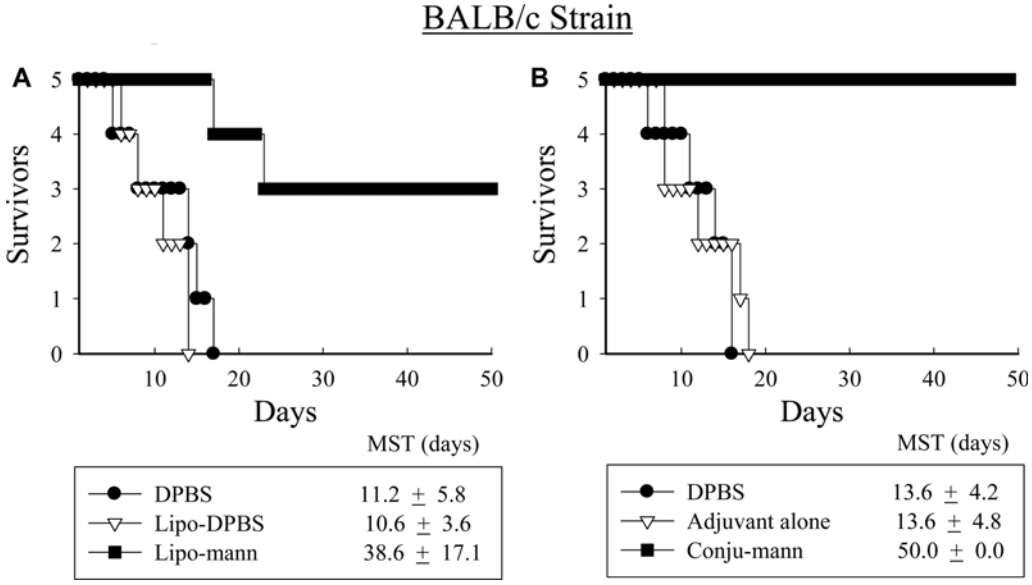

Fig. 1. The protective ability of Conju-mann is greater than Lipo-mann against disseminated candidiasis. Mean survival times (± S.E.) showed that Lipo-mann-immunized BALB/c mice survived longer than BALB/c mice that received no Lipo-mann ($p < 0.05$) (**A**). Three of the five mice survived until the end of the observation period. On the other hand, all of the five Conju-mann-immunized BALB/c mice survived during the period, whereas all negative control mice groups died within 16 and 18 days (**B**). The comparison of these survival rates indicate that the conjugate vaccine is more efficient and has greater protective ability than the liposomal vaccine. Note that Lipo-mann means that the mannan extract was encapsulated, and Conju-mann means that the mannan was conjugated with a protein (BSA) as a carrier.

Lipo-DPBS, respectively (Fig. 2A). In contrast to Lipo-mann, three out of five DBA/2J mice immunized with Conju-mann survived until the end of the 50 day observation period, whereas the unimmunized control DBA/2J mice groups all died within 9 days ($p < 0.05$) (Fig. 2B).

Protective effects of the two mannan vaccines in AKR strain against the disseminated disease

Although Lipo-mann-immunized AKR mice survived

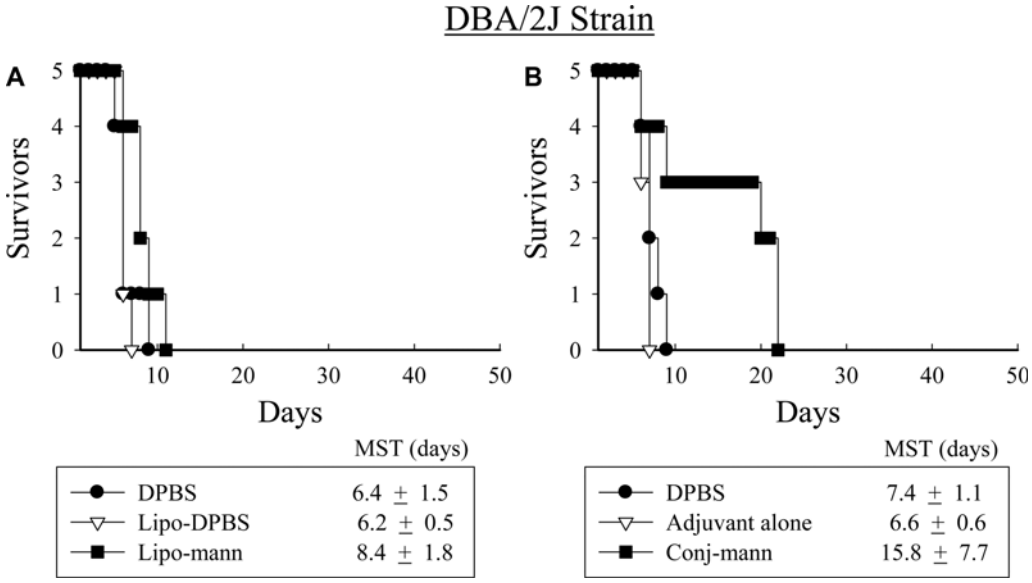

Fig. 2. Conju-mann formula but not Lipo-mann induces a partial protection in C5-deficient DBA/2J mice against disseminated candidiasis. The Lipo-mann formula was not protective, causing all of the three mice groups to have similar values of mean survival times (**A**). On the other hand, three of five DBA/2J mice immunized with Conju-mann survived during the entire period of 50 day observation period ($p < 0.05$) (**B**). Each group contained 5 mice. These data indicate that the complement system is essential and the conjugate formula is, once again, superior to the liposomal formula in the protection.

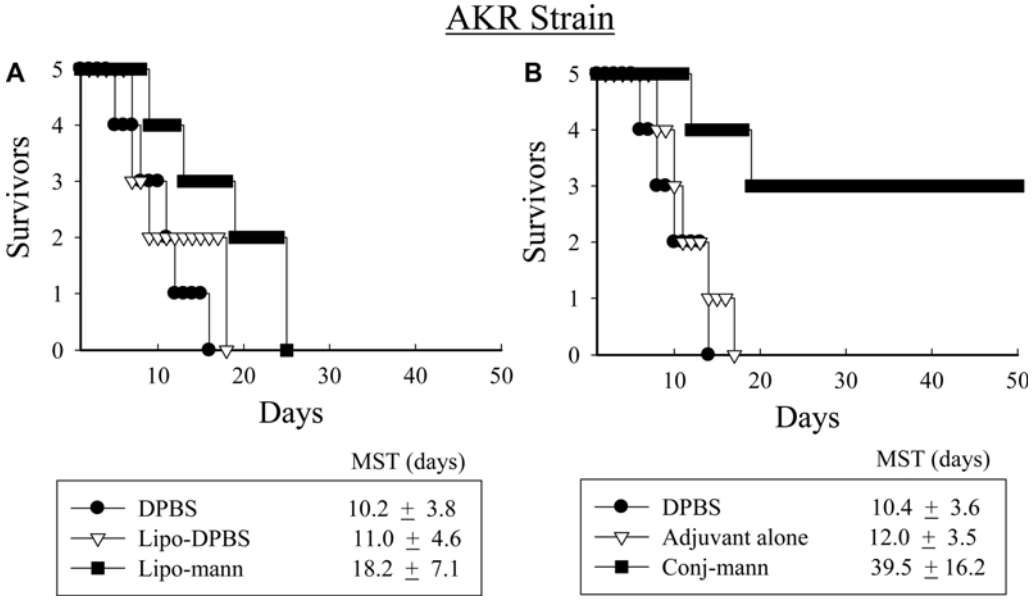

Fig. 3. The conjugate vaccine protects AKR mice against the disseminated disease, but Lipo-mann does not. Immunization of AKR mice with Lipo-mann slightly increased mean survival times as compared with the control AKR mice groups that received no Lipo-mann, but the immunized mice all died within 25 days (**A**). However, three of the five AKR mice immunized with Conju-mann survived during the entire 50 day observation period ($p < 0.05$). This observation confirms that the complement system is essential in protection. Also, the protection of AKR by the conjugate vaccine suggests that another protective mechanism is involved in the protection.

Table I. C5-deficiency caused no influence on induction of antibody production

	BALB/c	DBA/2J	AKR
Lipo-mann[a]	32[c]	32	32
Conju-mann[b]	32	32	32

[a]Lipo-mann: the mannan extract encapsulated into liposomes; [b]Conju-mann: the mannan extract conjugated to BSA as a carrier protein; [c]Values of antibody titer in arbitrary unit.

longer than DPBS- or Lipo-DPBS-given control AKR animals ($p < 0.05$), all of the immunized AKR animals died within 25 days, resulting in a very slight partial protection (Fig. 3A). However, three of the five AKR mice immunized with Conju-mann survived to the end of 50 day observation period (Fig. 3B). In contrast, all AKR control mice groups that received DPBS or adjuvant alone died, respectively, within 14 and 17 days (Fig. 3B).

Induction of antibody specific to the mannan

By the agglutination method, titers of the polyclonal antiserum samples obtained from the immunized mice with Lipo-mann or Conju-mann respectively were measured. Results analyzed from the titration showed that all of the three inbred mice groups that were immunized with the same kind of vaccine resulted in the same amount of antibody production specific to the mannan extract regardless of complement deficiency (Table I).

DISCUSSION

Our previous work showed that the two *Candida* mannan vaccines are protective in mice (BALB/c strain) with the normal complement system in their bodies against disseminated candidiasis due to *C. albicans* (Han and Cutler, 1995; Han et al., 1999). In comparing the number of immunizations, the Conju-mann formulation seems to be more efficient than the Lipo-mann formulation because Conju-mann protects mice through a priming dose followed by a single booster (Han et al, 1999), whereas Lipo-mann requires more than five immunizations for a proper antibody induction (Han and Cutler, 1995). The antigenic source for the two vaccines is same, but the method of antigen-delivery is different. That is, the antigen source is delivered by liposomes as a vehicle in one vaccine and by conjugation to the protein as a carrier in the other. There are a few other differences between the two vaccines, but until now, the superior formula for the protection against disseminated candidiasis has not been determined. Thus, we compared the effectiveness of the *Candida* mannan vaccines in the C5-deficient mouse model of disseminated candidiasis. This aim was not only to determine the involvement of complement in the protection by the vaccines but also to distinguish the superior formula for the protection.

We first tested the protective ability of the two formulae with the use of the BALB/c strain mice. As expected, both vaccines enhanced resistance of BALB/c mice with the complement in their bodies against the disseminated disease. However, the protective ability of the Conju-mann formula was much greater. For instance, there was a 100% protection of the mice that were immunized with Conju-mann during the entire observation period. In contrast, only 60% of Lipo-mann-immunized mice survived during the same period. These data indicate that the conjugate vaccine can be superior to the liposomal vaccine.

In order to compare the protective ability of the two formulations, we used two kinds of C5-deficient mouse strains - DBA/2J and AKR - instead of the BALB/c strain to determine the degree of protection. These experiments were also used to determine the role of complement in the protection by the two vaccines as well. As mentioned earlier, all of these strains are from inbred mice. With DBA/2J, the liposomal vaccine was not protective, whereas the conjugate vaccine slightly protected DBA/2J against disseminated candidiasis. However, the protection was partial and all of the Conju-mann-immunized mice died within 25 days although their survival times were longer than the control DBA/2J mice that received no conjugate vaccine. It was somewhat interesting that similar to the BALB/c strain, once again, the conjugate vaccine was superior to the liposomal vaccine-even though the survival rate of the Conju-mann-immunized DBA/2J mice was far lower than that of the BALB/c animals immunized with the same type of vaccine. We assumed that this may be due to the different susceptibility of the inbred mice. Others' data have mentioned that the degree of severity of the *C. albicans* infection varies and is dependent on the different mouse strains. DBA/2J strain is found to be most susceptible (Ashman et al., 1996, 2003). Therefore, to see whether or not our assumption was correct, we tested the AKR mouse strain that lacks C5 like the DBA/2J mice, but is more moderately susceptible to *C. albicans* than DBA/2J mice (Ashman et al., 1996). The data resulting from these experiments with the AKR strain show that the Lipo-mann vaccine slightly induced a partial protection and Conju-mann fully protected the AKR mice against disseminated candidiasis. In this case, 60% of the conjugate-immunized mice survived the entire 50 day observation period. Thus, these observations suggest that the protection provoked by our vaccine formulae may be influenced by the different

susceptibility of mouse strains. This explanation might be reasonable because the mean survival times (Fig. 2) from the control DBA/2J mice group that received no vaccine were shorter than mean survival times (Fig. 3) from the control AKR mice groups.

The implications of our data may be in agreement with those of Ashman's reports regarding the susceptibility issue as mentioned before. However, the main point here is that the complement system is essential to the protection by the mannan vaccines.

Next, to confirm our observation above that the complement system plays a role in the protection against disseminated candidiasis, we examined whether or not the C5-deficiency influences induction of the antibody production from the immunized mice. Resulting data showed that there was no reduction of the antibody production as determined by the mannan-coated beads. In other words, all of the three mouse strains induced the same level of antibody specific to the mannan extract. Therefore, it is assumed that the different degree of protection in C5-deficient strains is not a lack of anti-*Candida* antibody but lack of the component that needs to interact with the antibody.

In summary, we found that complement plays an important role in the protection by the two *Candida* vaccines, but the conjugate vaccine appears to be superior to the liposomal vaccine. Furthermore, the conjugate vaccine is protective under the condition of complement-deficiency. This led us to consider that the conjugate vaccine might have another protective mechanism such as cell-mediated immunity. Currently, this study is under investigation by using B-cell deficient mice.

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