Protection against Lethal Murine Coccidioidomycosis by a Soluble Vaccine from Spherules

C. ROGER ZIMMERMANN,1* SUZANNE M. JOHNSON,1 GREGORY W. MARTENS,1 ANDREW G. WHITE,1 BARBARA L. ZIMMER,1,2 AND DEMOSTHENES PAPPAGIANIS1

Department of Medical Microbiology and Immunology1 and Department of Dermatology,2 School of Medicine, University of California, Davis, California 95616

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The formaldehyde-killed, whole-spherule vaccine, which is protective against lethal challenge of laboratory animals with Coccidioides immitis, was fractionated. It yielded a soluble, multicomponent, subcellular fraction termed the 27K vaccine. This vaccine, when it was accompanied by adjuvant, protected mice against lethal intranasal and intravenous challenge with C. immitis.

Recovery from infection with Coccidioides immitis usually confers lifelong immunity to reinfection (33). Studies aimed at developing a protective anticoagulid vaccine indicated that the most efficacious nonviable vaccine was the formaldehyde-killed, whole-spherule (FKS) vaccine (12, 13, 20–25, 28, 29, 37). This vaccine protected against potentially lethal challenges with arthroconidia by the intranasal or intravenous routes in mice and by the respiratory route in monkeys. In humans, however, the maximum tolerable dose of the FKS was less than 1000th of the dose per kilogram of body weight that was protective in mice (27, 35). The low dose tolerated by humans may explain the lack of observed protective effect in a phase III field trial in humans (27). The present study was carried out to seek a less-irritating, yet protective, vaccine.

Kong et al. (18) found that the protective components were located primarily in the walls of mature spherules. An alkali-soluble, water-soluble extract of C. immitis mycelium prepared by Ward et al. (34), when it was administered with Freund's complete adjuvant, provided a significant level of protection in mice against intraperitoneal challenge and some measure of protection against intranasal challenge (20). Pappagianis et al. (28) mechanically extracted washed spherule walls and obtained a water-soluble extract which, when it was administered with Freund's or aluminum hydroxide (alum) adjuvants, provided a level of protection close to that afforded by FKS against a lethal intranasal challenge. More recently, Zimmer et al. (37) also extracted immunogens from the FKS vaccine. The results showed that immunizations with certain extracts and alum adjuvant were as protective as the FKS vaccine when mice were challenged intravenous with a lethal dose of arthroconidia.

Following the approach of Zimmer et al. (37), we have again shown that when a soluble, aqueous fraction (27K vaccine) prepared by mechanical disruption of the FKS vaccine was used with an alum adjuvant, it was nearly as protective as the parent FKS vaccine. The 27K vaccine was prepared from C. immitis Silveira (ATCC 28868). When suspended in sterile saline to a concentration of 3.5 mg/ml, the preparation was colorless, slightly opalescent, and virtually devoid of intact microscopically visible fragments. Both protein (26) and carbohydrate (presumably polysaccharides) (32) were present in the 27K vaccine.

Groups of seven 16- to 20-g Swiss Webster mice were injected with 0.2 to 0.4 ml of 27K vaccine, with or without alum adjuvant (Cutter Laboratories, Berkeley, Calif.) or with alum alone. At 1-week intervals, we administered subcutaneously three doses consisting of either 1 mg of an individual vaccine alone, 1 mg of an individual vaccine with 4 mg of alum, or 4 mg of alum alone. Four weeks after the third dose, the mice were challenged with C. immitis arthroconidia (Silveira strain; ATCC 28868) intravenously in a tail vein or intranasally. The experiment was terminated 13 weeks after the challenge; the survivors were sacrificed, and the whole lungs, liver, and spleen of each survivor were cultured on Mycobiotic agar (Difco, Detroit, Mich.). C. immitis was recovered from at least one of the organs cultured from each surviving mouse. Survival differences between groups of mice receiving different vaccines and alum were compared by the Mantel-Haenszel log rank test of significance. A P of <0.05 was considered significant.

Mouse survival in the protection experiments is shown in Fig. 1. The intranasal challenge was virtually as rigorous as the intranasal challenge. When analyzed statistically, the survival of mice immunized with the 27K vaccine with alum was significantly different from that of mice injected with alum alone when they were challenged intravenously with 500 (P = 0.007) and 5,000 (P = 0.0002) arthroconidia and intranasally with 5,000 (P = 0.003) and 15,000 (P = 0.04) arthroconidia. Similar results were obtained when the survival of the groups of mice immunized with the 27K vaccine alone was compared to the survival of the groups of mice injected with alum alone (5,000 arthroconidia intranasally, P = 0.002; 500 and 5,000 arthroconidia intravenously, P = 0.007 and 0.0002, respectively). There was no significant difference between results with the 27K vaccine alone and those with alum alone in mice challenged with 15,000 arthroconidia intranasally (P = 0.17). Also, with the 500-arthroconidia intranasal challenge, there were no significant differences in the levels of protection given by the three vaccines versus those given by alum alone (FKS and 27K with alum, P = 0.14; 27K alone, P = 0.73).

In order to resolve the components of the 27K vaccine, 50-µg aliquots of the 27K vaccine were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under re-
ducing conditions with a 12% gel and accompanying molecular weight standards (Bio-Rad, Hercules, Calif.) (19). As shown in Fig. 2, the Coomassie blue-stained material was not separated into a pattern of discrete bands but rather took the form of a continuous smear extending the length of the gel. Interestingly, when similar gels were blotted onto nitrocellulose (14) and reacted with rabbit serum and an anti-27K vaccine rabbit serum, a few bands were resolved from the gel-long smear of reacting antigens (Fig. 3, lane 2). In Fig. 4 to 6, the 27K vaccine was fractionated by isoelectric focusing (IEF) in a 5.0% polyacrylamide gel containing 2.4% ampholytes (pH 3.5 to 10; Bio-Rad) at 200 V for 2 h with accompanying molecular pI standards (Bio-Rad) (6). The precipitated bands of two identical lanes were photographed with oblique light without additional staining (Fig. 4). Both unstained (Fig. 4) and Coomassie blue-stained (Fig. 5, lane 2) IEF gels of the 27K vaccine yielded discrete bands. In addition, when replicate gels were blotted onto nitrocellulose (14) and reacted with rabbit serum and an anti-27K vaccine rabbit serum, discrete bands were resolved (Fig. 6, lane 2).

In the experiments presented here, the 27K vaccine mixed with alum provided protection equal to that of the FKS vaccine against lethal challenge in mice. Alum was used in this study because it had shown enhancement of the protection of the earlier soluble vaccine (28) and, in an initial study, of the 27K vaccine (37). In addition, its use was based on the long-approved adjuvant in vaccines in clinical use for humans. There is now, however, a general impression that alum is a poor adjuvant for the induction of cell-mediated immunity, which appears central to resolution of *Coccidioides* infection.

FIG. 1. Survival of vaccinated mice following intranasal or intravenous challenge by arthroconidia. Groups of seven mice were immunized and challenged with 500, 5,000, or 15,000 arthroconidia.

FIG. 2. Reduced sodium dodecyl sulfate–12% polyacrylamide gel with the 27K vaccine after Coomassie blue staining.

FIG. 3. Lane 2, reacting antigens blotted onto nitrocellulose and reacted with rabbit serum and an anti-27K vaccine rabbit serum.

FIG. 4. IEF gel of the 27K vaccine stained with Coomassie blue.

FIG. 5. IEF gel of the 27K vaccine unstained.

FIG. 6. Replicate gels blotted onto nitrocellulose and reacted with rabbit serum and an anti-27K vaccine rabbit serum.
and of immunity to C. immitis (1–5, 7–11, 17, 31). Although our results do not allow discrimination between antibody- and cell-mediated immunity, the ability of alum to enhance protection by the 27K vaccine raises the possibility that antibodies play a role in protection (27, 30, 36).

Optimal immunization by the 27K vaccine may require the addition of adjuvants that can enhance cell-mediated immunity, a function that may be inherent in the FKS vaccine. Future studies will make use of newer adjuvants, such as the RIBI (RIBI ImmunoChem Research, Hamilton, Mont.) preparations that favor Th1 responses, which may prove to enhance the immunogenicity of the 27K vaccine or its derivatives. Alternatively, the polysaccharides that are present in the 27K vaccine may themselves provide critical antigenic or adjuvant effects. Future studies will address the possibility that endogenous chitin is an important adjuvant for the protective immunity obtained with the FKS or 27K vaccine (15, 16). IEF gel fractionation of the 27K vaccine indicated the presence of several components. The protective components will have to be identified. Enhancement of the immunogenicity may require isolation of particularly active components and their synthesis by molecular genetic methods.

**FIG. 3.** Immunoblots of gel lanes identical to that shown in Fig. 2 after being reacted with normal, prevaccination serum (lane 1) and anti-27K vaccine serum (lane 2).

**FIG. 4.** Unstained 5% IEF gel (pH 3.5 to 10) of the 27K vaccine.

**FIG. 5.** Coomassie blue-stained 5% IEF gel (pH 3.5 to 10) of the 27K vaccine. Lane 1, molecular pl standards; lane 2, 27K vaccine.

**FIG. 6.** Immunoblots of gel lanes identical to those shown in Fig. 5 after being reacted with normal, prevaccination serum (lane 1) and anti-27K vaccine serum (lane 2).
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