

Protection of Mice against *Coccidioides immitis* Intranasal Infection by Vaccination with Recombinant Antigen 2/PRA

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Subcutaneous vaccination with recombinant antigen 2/PRA (rAg2/PRA) protected BALB/c mice against intranasal infection with *Coccidioides immitis*. Subcutaneously vaccinated C57BL/6 mice and intranasally vaccinated BALB/c mice were protected against larger numbers of infecting spores. Weight loss correlated with lethality, but histologic appearance did not. These studies support rAg2/PRA vaccination to prevent coccidioidomycosis.

Although *Coccidioides immitis* can cause life-threatening infections (9, 13, 21–23), most resolve without treatment and induce long-term immunity (2, 3, 6, 10, 24), suggesting that a vaccine might be feasible. One recombinant antigen has been referred to historically as “antigen 2” (14) or, more recently, as “PRA,” a deglycosylated proline-rich antigen purified from spherules (7, 12). The sequences of antigen 2 and PRA are identical (8, 25), and in this study, the term “antigen 2/PRA” (Ag2/PRA) will be used for both. Three laboratories have reported protection by subcutaneous vaccination with recombinant Ag2/PRA (rAg2/PRA) against intraperitoneal (i.p.) infection with *C. immitis* (1, 15, 18). Here, we evaluate protection against the more stringent respiratory infection (20).

rAg2/PRA (1) was used with either an oil emulsion of monophosphoryl lipid A (MPL-SE) for subcutaneous injection or aqueous MPL (MPL-AF) for intranasal administration (Corixa, Inc., Hamilton, Mont.). Whole-spherule vaccine was prepared from 96-h growths of arthroconidia in liquid Converse medium, resulting in mature endospore spherules (11, 12). After exposure to 5% formalin, washed spherules were lyophilized.

Female, 6-week-old BALB/c and C57BL/6 mice (Jackson Laboratories, Bar Harbor, Maine; or Harlan-Sprague-Dawley, Indianapolis, Ind.) were vaccinated in groups of 10 to 14 animals either subcutaneously (1) or intranasally (20 μ l of vaccine was delivered to the nares during anesthesia with i.p. ketamine-xylazine) and repeated 4 weeks later. Mice immunized with killed spherules were injected subcutaneously on days 0, 7, and 14 with 1 mg per dose (26).

One month after vaccinations, anesthetized mice were infected intranasally with arthroconidia (*C. immitis* strain RS) suspended in 50 μ l of sterile saline. The infecting inoculum size was determined by colony enumeration on subculture. Moribund animals and mice surviving 56 days were euthanized by anesthetic overdose, and the severity of infection was assessed (Table 1). Lungs and spleens of all mice were removed for

qualitative culture to confirm growth of fungus. Other tissues were collected as indicated for histopathologic examination. Differences in survival among groups were analyzed by the Kaplan-Meier (product limit) method with the log-rank Mantel-Haenszel test (16). Differences with *P* values of 0.05 or less, without correction for repeated measurements, were taken as significant. Other comparisons utilized either Kruskal-Wallis or chi-square tests, as indicated in the results.

The degree of protection afforded BALB/c mice by rAg2/PRA vaccination showed a striking dependence upon size of the infecting inoculum (Fig. 1A). Whereas significant protection was evident when vaccinated mice were infected with seven arthrospores, protection was lost if a larger inoculum was used. With a relatively small inoculum, it is possible that some mice do not receive a single arthroconidium beyond the upper airway. In mice receiving adjuvant only and challenged with seven arthroconidia, only 4 out of 24 (17%) survived to the end of the studies and at necropsy had neither gross lesions nor organisms cultured from lungs or spleens. In comparison, 20 of 34 (59%) surviving animals vaccinated with rAg2/PRA showed no evidence of infection at necropsy (*P* = 0.0025; Fisher exact test). This difference suggests that vaccination with rAg2/PRA may also have reduced the rate of infection.

Because the consistent intranasal delivery of small numbers of arthroconidia is technically difficult, we evaluated the inoculum effect in another susceptible mouse (Fig. 1B) (17). Unvaccinated C57BL/6 mice were nearly as sensitive to lethal infection as BALB/c mice. However, vaccinated C57BL/6 mice resisted infection with as many as 145 arthroconidia. Inspection of the dose-response curves in Fig. 1A and B demonstrates that vaccination with rAg2/PRA induces significantly prolonged survival at an approximately 20-fold-higher infecting inoculum in C57BL/6 mice compared to BALB/c mice.

As another approach, BALB/c mice were immunized intranasally instead of subcutaneously (Fig. 1C). Intranasal vaccination produced significant prolongation of survival, with infecting inocula as large as 97 spores (*P* = 0.005).

Comparative vaccination of BALB/c mice with whole killed spherules allowed 90 to 100% of mice to survive to 56 days with infecting inocula as high as 84 arthroconidia per mouse (Fig.

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TABLE 1. Criteria for assessing the extent of infection as evidenced by necropsy examination

Disease score	Infection site characteristic ^a	
	Lung	Abdomen
0	No gross disease	No gross disease
1	1-2 granulomas in lungs	1-2 granulomas—mesentery, spleen, or liver
2	3-8 small, well-defined granulomas in lungs	3-8 granulomas—mesentery, spleen, or liver
3	Multiple large or coalescing granulomas, unilateral adhesions	Extensive, coalescing granulomas in mesentery
4	Massive bilateral granulomas, adhesions, <30% normal lung tissue	Extensive granulomas and adhesions, severe peritonitis

^a The number of granulomas was determined without sectioning.

1D). However, protection was lost if the infecting inoculum was sufficiently high. Moreover, vaccination with whole killed spherules resulted in granulomas ranging from 0.5 to 2.0 cm in diameter at the injection site. This is much more inflammatory than subcutaneous vaccination with rAg2/PRA, which caused no observable induration. For C57BL/6 mice vaccinated with

whole killed spherules, survival of all mice was observed at a dose of 250 arthroconidia (data not shown).

In BALB/c mice, regardless of the vaccine group, weight loss was significantly associated with fatality. Mice that died before 56 days lost 3 to 8 g of weight during the course of infection, whereas 74% of surviving mice lost 2 g of weight or less in spite

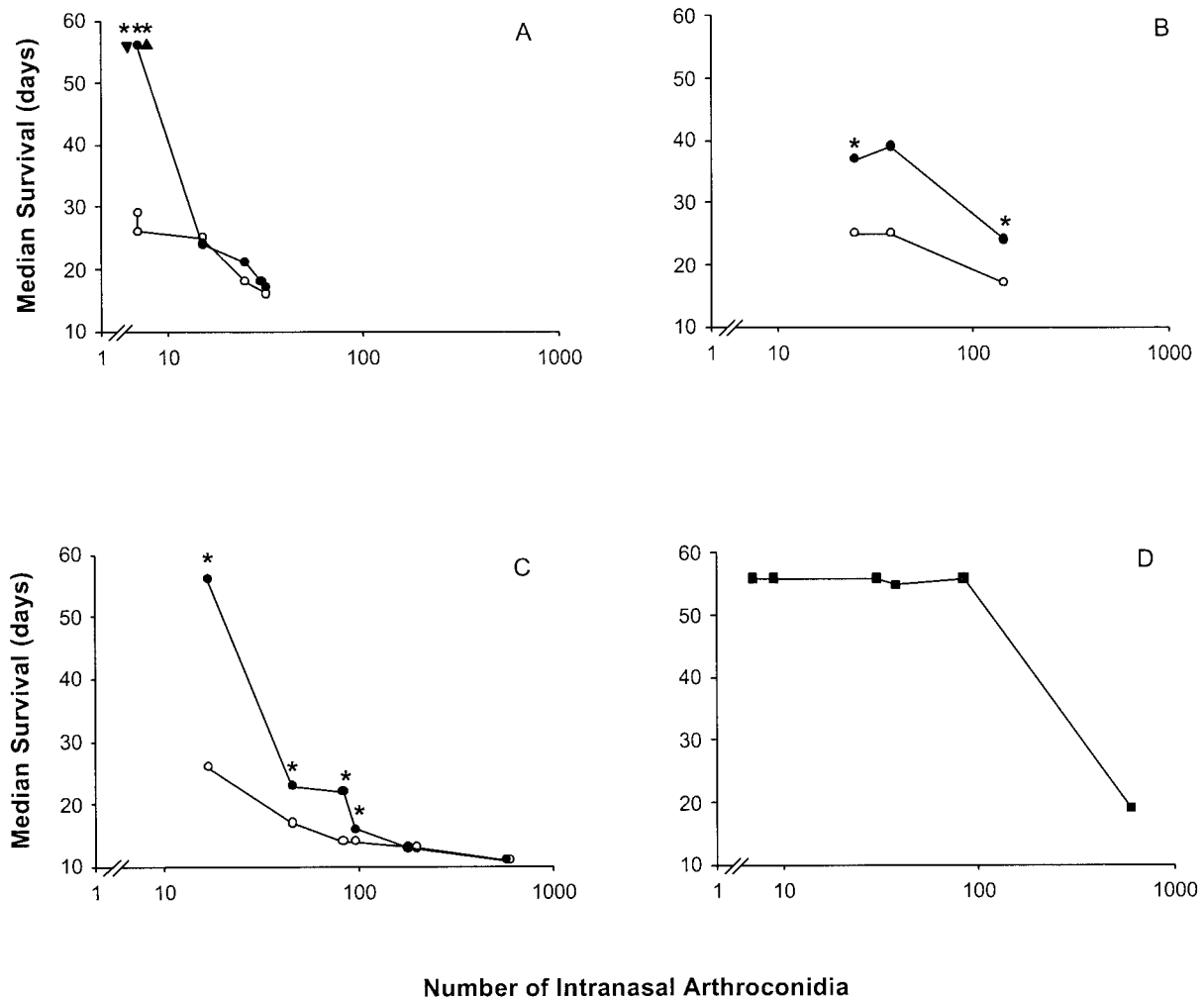


FIG. 1. (A to C) Median survival of vaccinated mice after intranasal infection with *C. immitis*. Vaccinations were adjuvant only (open symbols), 0.5 µg of rAg2/PRA (solid inverted triangle), 1.0 µg of rAg2/PRA (solid circles), 5.0 µg of rAg2/PRA (solid upright triangle), or 1.0 mg of whole killed spherules (solid squares). Each point indicates results from groups ranging from 10 to 14 animals. Each asterisk indicates a significant difference ($P < 0.05$) between a specific group of animals receiving rAg2/PRA as compared to a group administered the same number of arthroconidia, but which received adjuvant without rAg2/PRA. (A) BALB/c mice vaccinated subcutaneously. (B) C57BL/6 mice vaccinated subcutaneously. (C) BALB/c mice vaccinated intranasally. (D) BALB/c mice vaccinated with whole killed spherules.

of extensive lung pathology observed at termination. The difference in weight of fatalities versus survivors was highly significant (Kruskal-Wallis test, $P < 0.001$). In contrast, among 55 mice that survived to 56 days, 19 exhibited grade 3 or 4 pulmonary pathology. In addition, the spleens of all mice were at least four times normal size, and some mice had granulomas visible in the liver or spleen parenchyma. These findings were indistinguishable from those in animals who died before 56 days. The surviving C57BL/6 mice had a disease pattern similar to that seen in BALB/c mice.

These studies demonstrate protection against intranasal infection by vaccination with rAg2/PRA. Although protection was evident in BALB/c mice with as little 0.5 μg of rAg2/PRA per vaccine dose, protection from subcutaneous vaccination was lost when more than seven arthroconidia were used for infection. In C57BL/6 mice, protection could be detected with 20-fold-larger numbers of arthroconidia. This is an important technical finding that favors the use of C57BL/6 mice for future testing of coccidioidal vaccine candidates. Moreover, intranasal vaccination of BALB/c mice with rAg2/PRA afforded significant prolongation of survival, with inoculum sizes as large as 97 spores. These findings provide further substantiation of the protection engendered by rAg2/PRA and support the use of an intranasal route of immunization for future vaccine development. Intranasal vaccination has afforded protection against veterinary diseases (4, 5, 19) and conceivably could be an effective mode of administration against coccidioidomycosis. The current findings clearly support the further evaluation of rAg2/PRA as a vaccine candidate to prevent coccidioidomycosis.

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