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Considerable Differences in Vaccine Immunogenicities and Efficacies Related to the Diluent Used for Aluminum Hydroxide Adjuvant▽

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We are developing an anticandidal vaccine using the recombinant N terminus of Als3p (rAls3p-N). We report that although more rAls3p-N was bound by aluminum hydroxide diluted in saline than by aluminum hydroxide diluted in phosphate-buffered saline (PBS), its immunogenicity and efficacy were superior in PBS. Thus, protein binding, by itself, may not predict the efficacy of some vaccines with aluminum adjuvants.

Hundreds of millions of doses of aluminum adjuvants have been administered to humans over the past century (5, 11, 12). One standard formulation is Alhydrogel, a gelatinous matrix of aluminum hydroxide, which minimizes lot-to-lot variability and enables facile adsorption of proteins (5, 12). As noted by Hem et al., aluminum hydroxide in its dehydrogenated, crystalline form is chemically aluminum oxyhydroxide [AlO(OH)] (7), but in its aqueous phase, it acquires an additional water molecule to become aluminum trihydroxide [$Al(OH)_3$].

We have developed a vaccine using the recombinant N terminus of the candidal adhesin Als3p (rAls3p-N), which has been shown to protect mice against otherwise-lethal disseminated candidiasis (8, 9, 15, 16). The vaccine is also effective in combination with Alhydrogel diluted in phosphate-buffered saline (PBS) (8). Vaccine efficacy requires an adjuvant, as the vaccine does not significantly improve survival if no adjuvant is utilized. To determine the optimal diluent for the continued development of the rAls3p-N vaccine, we assessed its relative binding to $Al(OH)_3$, its immunogenicity, and its efficacy in the presence of PBS versus in the presence of saline.

rAls3p-N (amino acids 17 to 432 of Als3p) was produced in *Saccharomyces cerevisiae* and purified by Ni-nitrilotriacetic acid matrix affinity purification as previously described (15). The isoelectric point of several batches of rAls3p-N was determined to be 7.1 using a Ready Gel IEF pH 5-to-8 gel (Bio-Rad) per the manufacturer's instructions (data not shown).

To quantify protein binding to $Al(OH)_3$, rAls3p-N was diluted to 0.2 mg/ml in saline (pH 6.5, 0.9% sodium chloride) or PBS (pH 7.40, 0.9% sodium chloride, 0.1% sodium-potassium phosphate), with or without 0.13 mg/ml of Alhydrogel (Brenntag Biosector, Frederikssund, Denmark). The solutions were vortexed, incubated for 30 min at room temperature, and centrifuged at 13,000 rpm for 10 min on a tabletop microcentrifuge. The protein concentration in the supernatant (i.e., unbound) was calculated by UV absorption as follows: (concentration without Alhydrogel – concentration with Alhydrogel)/(concentration without Alhydrogel). Protein binding was found to be slightly but significantly higher in the presence of saline than in the presence of PBS (median [75th, 25th quartiles] = 55% [57%, 53%] versus 50% [53%, 44%] [quadruplicate experiments]; $P = 0.04$ [Mann-Whitney U test]).

To determine immunogenicity, female BALB/c mice (≥6 months old; Charles River Laboratories) were immunized by subcutaneous injection of PBS-Alhydrogel (P-A), PBS–rAls3p-N–Alhydrogel (P-3), saline-Alhydrogel (S-A), or saline–rAls3p-N (300 μg)–Alhydrogel (S-3). The Alhydrogel and rAls3p-N doses were 200 and 300 μg in 0.2 ml total, respectively, based on optimal concentrations determined in prior efficacy studies (data not shown). For all rAls3p-N batches, endotoxin levels were ≤0.06 nanogram per dose, as determined by a *Limulus* amoebocyte assay (Charles River Laboratories). All procedures involving mice were approved by the institutional animal use and care committee and followed the National Institutes of Health guidelines for animal housing and care (12a).

Splenocytes from vaccinated or control mice were harvested at 3 weeks and stimulated for 4 days with rAls3p-N at 50 μg/ml or tetanus toxoid (Cylex Inc., Columbia, MD) at 2 limits of flocculation per ml (negative control) in 96-well plates, as described previously (17). From some of the wells, 50 μl of the supernatant was harvested for cytokine analysis using the murine type 1/type 2 cytometric bead array kit (BD Biosciences Pharmingen) per the manufacturer's instructions. Brefeldin A was added to the wells for the last 4 h of culture. The cells were fixed, permeabilized, and stained intracellularly for CD4, gamma interferon (IFN-γ), and interleukin-4 (IL-4), as we have previously described (17). Three-color flow cytometry was performed on a Becton Dickinson FACScan instrument.

rAls3p-N stimulation of the splenocytes from the P-3-vaccinated mice generated significantly higher frequencies of Th1 ($CD4^+$ IFN-γ^+ IL-4^-) and Th2 ($CD4^+$ IFN-γ^- IL-4^+) cells than those in all other groups (Fig. 1A). Similarly, the supernatant from rAls3p-N-stimulated splenocytes from the P-3-vaccinated mice contained significantly elevated levels of tumor necrosis factor, IFN-γ, IL-4, and IL-5 compared to levels in the supernatant from P-A splenocytes (Fig. 1B). The supernatant from P-3 splenocytes contained significantly more tu-

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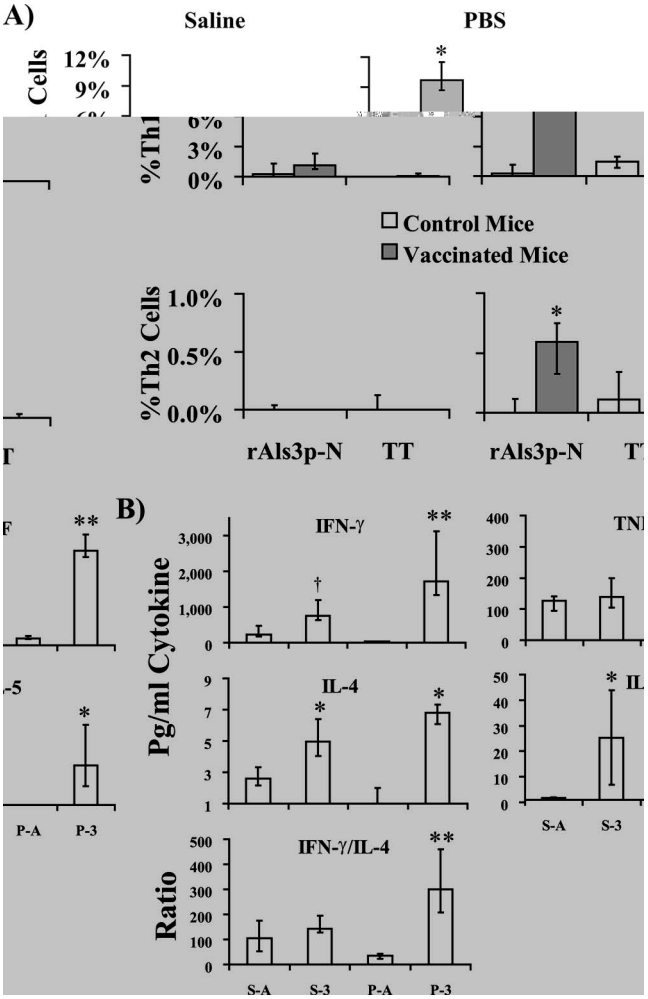

FIG. 1. Immunogenicity of S-3 versus that of P-3. (A) Frequency of Th1/Th2 lymphocytes among ex vivo-stimulated splenocytes from BALB/c mice (eight mice per group) vaccinated with S-A, S-3, P-A, or P-3. Three weeks after the vaccination, the splenocytes were harvested and stimulated for 4 days with rAls3p-N or tetanus toxoid (TT). Medians and interquartile ranges are shown. *, P was ≤0.05 versus all other groups. (B) Culture supernatant cytokines induced by rAls3p-N stimulation of the splenocytes from the vaccinated or control mice. **, P was ≤0.03 versus all other groups; *, P was ≤0.05 versus S-A or P-A controls; †, P was 0.08 versus S-A.

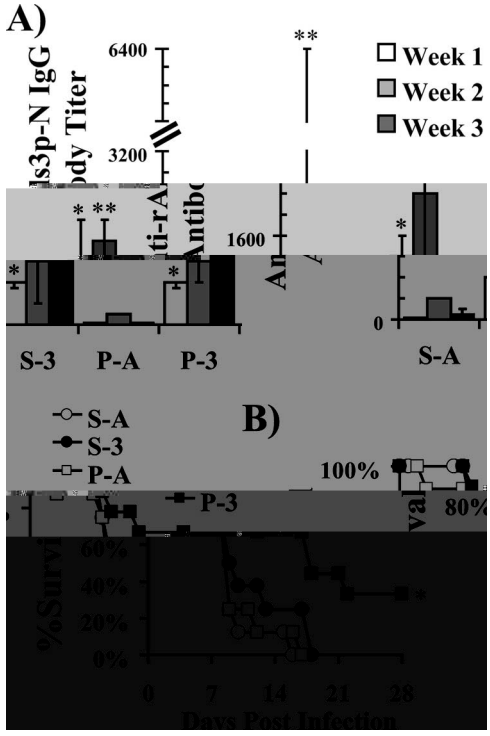

FIG. 2. Antibody titers and efficacy of the vaccine in the presence of saline versus in the presence of PBS. (A) Serum immunoglobulin G (IgG) anti-rAls3p-N titers were determined by enzyme-linked immunosorbent assay 1, 2, and 3 weeks after vaccination of BALB/c mice with S-A, S-3, P-A, or P-3 (eight mice per group). Medians and interquartile ranges are shown. *, P was <0.05 versus the adjuvant control by the Mann-Whitney U test; **, P was <0.05 versus the adjuvant control by the Mann-Whitney U test and versus week 1 titers by the signed-rank test. (B) Three weeks after vaccination, mice were infected via the tail vein with 2.7×10^5 blastospores of *C. albicans* SC5314. *, P was <0.04 versus both P-A and S-3.

mor necrosis factor and IFN-γ and exhibited a trend toward more IL-4 ($P = 0.055$) than the supernatant from S-3 splenocytes.

Serum antibody titers from 1, 2, and 3 weeks postvaccination were determined by enzyme-linked immunosorbent assay of 96-well plates coated with 5 μg/ml rAls3p-N, as we have described previously (9, 16). While the antibody titers increased each week in vaccinated mice, the titers did not differ at any time point between the mice vaccinated with S-3 and those vaccinated with P-3 (P was 0.8, 0.5, and 0.6 at 1, 2, and 3 weeks, respectively) (Fig. 2A).

Finally, to determine vaccine efficacy, the vaccinated and control mice were infected via the tail vein with *Candida albicans* SC5314, as we have previously described (8, 9, 14–17). Mice vaccinated with P-3 had significantly improved survival compared to all other groups (Fig. 2B). Mice vaccinated with S-3 did not have significantly improved survival compared to mice receiving S-A.

It has been established that optimal binding of protein to $Al(OH)_3$ occurs when the pH of the diluent matrix is between the isoelectric point of the protein in question and the point of zero charge of the aluminum salt (11–13). Because the isoelectric point of rAls3p-N was similar to the pH levels of saline and PBS, it is not surprising that the levels of protein binding were similar in both diluents. Maximum protein binding has been hypothesized to predict superior vaccine immunogenicity and efficacy (1, 11, 12). In contrast, several studies have previously reported that antibody titers resulting from vaccination are independent of protein binding to aluminum (2, 3, 10). Furthermore, Hansen et al. found that phosphorylation of a protein antigen decreased the strength of binding to $Al(OH)_3$ and increased proliferative T-cell responses to the protein (6). Our study is concordant with these results but is unique in evaluating the impact of aluminum adjuvant diluent on T-cell cytokine responses and vaccine efficacy measured by survival after infectious challenge. The precise mechanism by which the immunogenicity of the rAls3p-N vaccine was enhanced in PBS versus in saline is unknown, although functions of aluminum

aside from its depot effects (4), differences in strength of adsorptive force (as opposed to absorptive capacity) (6), or altered binding of protein to host mannose receptors (18) in different pH environments may play a role.

Collectively, these data indicate that protein binding, by itself, may not predict the efficacy of some vaccines with aluminum adjuvants. These data also support the use of PBS as a diluent in ongoing efforts to develop the rAls3p-N–Alhydrogel vaccine.

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