

## Therapy of Murine Pulmonary Aspergillosis with Antibody-Alliinase Conjugates and Alliin<sup>∇</sup>

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*Aspergillus fumigatus* is an opportunistic fungal pathogen responsible for invasive aspergillosis in immunocompromised individuals. The high morbidity and mortality rates as well as the poor efficacy of antifungal agents remain major clinical concerns. Allicin (diallyl-dithiosulfinate), which is produced by the garlic enzyme alliinase from the harmless substrate alliin, has been shown to have wide-range antifungal specificity. A monoclonal antibody (MAb) against *A. fumigatus* was produced and chemically ligated to the enzyme alliinase. The purified antibody-alliinase conjugate bound to conidia and hyphae of *A. fumigatus* at nanomolar concentrations. In the presence of alliin, the conjugate produced cytotoxic allicin molecules, which killed the fungus. *In vivo* testing of the therapeutical potential of the conjugate was carried out in immunosuppressed mice infected intranasally with conidia of *A. fumigatus*. Intratracheal (i.t.) instillation of the conjugate and alliin (four treatments) resulted in 80 to 85% animal survival (36 days), with almost complete fungal clearance. Repetitive intratracheal administration of the conjugate and alliin was also effective when treatments were initiated at a more advanced stage of infection (50 h). The fungi were killed specifically without causing damage to the lung tissue or overt discomfort to the animals. Intratracheal instillation of the conjugate without alliin or of the unconjugated monoclonal antibody significantly delayed the death of the infected mice, but only 20% of the animals survived. A limitation of this study is that the demonstration was achieved in a constrained setting. Other routes of drug delivery will be investigated for the treatment of pulmonary and extrapulmonary aspergillosis.

*Aspergillus fumigatus* is an opportunistic fungal pathogen that is responsible for invasive aspergillosis (IA) in immunocompromised individuals (19, 22, 25). Patients with hematological or solid malignancies, as well as organ transplant recipients, are particularly vulnerable to infection. Pulmonary infection by *A. fumigatus* airborne conidia is the predominant cause of IA (22). Despite advances in early diagnosis and new antifungal agents, IA currently remains a leading cause of death in the immunocompromised patient population, with an attributable mortality rate ranging from 30% to 80% (13, 50).

Alliin (diallyl-dithiosulfinate), the biologically active molecule of garlic, has been shown to have a very wide range of antimicrobial activities and contributes to the defense of the garlic plant against soil microorganisms (1, 11, 15, 20, 29, 36, 44). Allicin is produced by the catalytic reaction of the enzyme alliinase (EC 4.4.1.4) with the inert, nonprotein amino acid substrate alliin [(+)-S-allyl-cysteine sulfoxide]. Crushing the garlic clove breaks down the compartmentalization and brings the enzyme and its substrate into contact, leading to allicin production (20, 30). The potential use of pure allicin as an anti-*Aspergillus* agent *in vivo* was shown in our previous work (44). Despite its short half-life, five repetitive doses of pure allicin administered intravenously (i.v.) to mice infected with *A. fumigatus* significantly prolonged their survival. The delivery of allicin, however, remains a major concern, due to its insta-

bility in blood circulation. Allicin rapidly transforms into secondary products that lack antimicrobial activity following intravenous injection (14, 20, 37).

Our novel approach for antifungal therapy overcomes this problem by generating the production of allicin on the targeted pathogen. In a previous investigation, we developed a system of targeted production of allicin to kill specifically cancer cells (3, 27). In the present study, the potential efficacy of this novel *in vivo* treatment was investigated with a murine model of invasive pulmonary aspergillosis (IPA) (54). We prepared a conjugate consisting of the alliinase enzyme ligated to a monoclonal anti-*A. fumigatus* antibody to target the production of allicin molecules to the surface of the fungus. After infection, the conjugate and then the substrate alliin were repeatedly administered by intratracheal (i.t.) instillation as described previously (17). The main advantages of this approach over other antibody-directed enzyme prodrug therapy (ADEPT) systems (4) are (i) the harmless nature of the prodrug alliin, a natural food component that has been declared by the FDA as a substance that is generally recognized as safe (GRAS) and that can be administered in unlimited amounts and (ii) the fact that the hydrophobic allicin molecules produced on the target cell have a limited area of effect; due to their high reactivity and short lifetime, they kill the fungi without causing visible damage to the adjacent lung epithelial cells. To the best of our knowledge, this work constitutes the first example of a targeted allicin generation system for antimicrobial treatment.

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## MATERIALS AND METHODS

**Fungal strains.** *A. fumigatus* strain 293 and the clinical isolate CBS 144.89 (a gift from Jean-Paul Latgé, Aspergillus Unit, Pasteur Institute, Paris, France) were used for *in vitro* experiments. The fluorescent strain CBS 144.89/DsRed, previously described (54), was used as an infection readout in mice. Resting conidia were counted with a hemacytometer and grown in RPMI-MOPS (44). Other fungal strains tested for the binding of the anti-*A. fumigatus* monoclonal antibody (MAb) MPS5.44 (see below) were *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus terreus*, *Candida albicans*, *Candida krusei*, and a *Mucor* mold.

**Preparation of pure alliin.** Pure alliin was produced by passing a solution of synthetic, nature-identical alliin (see below) through an immobilized alliinase column (30). Alliin was analyzed and quantified by high-pressure liquid chromatography (HPLC), as described previously (28).

**Preparation of the MAb-alliinase conjugates.** Alliinase was purified from garlic cloves as previously described (38, 45, 46). Anti-*A. fumigatus* MAbs were produced in mice. A preparation containing freshly harvested AF293 conidia and hyphae served as the antigen. Hybridomas were screened for binding to AF293 hyphae. Clone MPS5.44, IgM isotype, was selected for further study. The binding of this antibody to several types of mammalian cell monolayers was at least one order of magnitude lower than its binding to *A. fumigatus*. The IgM antibodies were purified from the hybridoma cell culture medium by affinity chromatography on mannan binding protein (MBP) columns (Pierce, Rockford, IL). As a control, we used a nonrelevant, anti-dinitrophenol IgM MAb and purified it as described for MPS5.44.

Conjugation of MAbs with alliinase was performed in three steps: (i) thiolation of the MAbs with iminothiolane according to the method of Lambert et al. (21); (ii) derivatization of alliinase with NHS-PEO<sub>4</sub>-maleimide, and (iii) conjugation of the two modified proteins according to the manufacturer's protocol (Pierce, Rockford, IL). The molar ratio of MAb/alliinase taken for conjugation was 1:3. The high-molecular-weight (MW) conjugates (MW of ~1,200) were separated from free alliinase (MW of 100) by size exclusion chromatography on a Superdex 200 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

Alliinase activity of the fractions was determined using the NTB (2-nitro 5-thiobenzoic acid) method as described previously (28). Fractions that contained IgM and possessed the highest levels of alliinase activity were pooled and used as the purified IgM-alliinase conjugate. Synthetic, nature-identical alliin ( $\alpha = +63^\circ$ ) was used as the substrate. It was synthesized from L-cysteine and allyl-bromide and subjected to H<sub>2</sub>O<sub>2</sub> oxidation as described previously (51). A unit (U) of alliinase activity was defined as the amount of enzyme required to release 1  $\mu$ mol of pyruvate per min (28). The chemical conjugation did not impair alliinase activity. The specific activity of alliinase and of the conjugates was found to be 100 U/nmol and 125 U/nmol, respectively. The dominant population of the conjugate molecules, as determined by dynamic light scattering, had a mean hydrodynamic size of approximately 100 nm. The time autocorrelation function was determined by use of the CONTIN algorithm (35). Assuming a spherical shape for the conjugate and an average density of 1.2 g/cm<sup>3</sup>, a diameter of 120 nm would be expected.

**Binding of MAbs and conjugates to *A. fumigatus* (ELISA).** The binding of the MAb-alliinase conjugate or the unconjugated MPS5.44 antibodies to either *A. fumigatus* hyphae or swollen conidia was performed in 96-well plates in triplicates. A nonspecific IgM antibody (anti-2,4-dinitrophenol [DNP]) served as a negative control. Blocking of nonspecific binding was carried out by preincubation (37°C, 1 h) of the conidia or hyphae with a solution of hemoglobin (1%) in TBST (20 mM Tris, pH 8.0; 140 mM NaCl, 0.05% Tween 80). Plates were then washed twice with TBST and incubated with serial dilutions of the above antibodies in TBS (20 mM Tris, pH 8.0; 140 mM NaCl) for 30 min. Unbound antibodies were removed by four consecutive washings with TBST and incubated (room temperature [RT], 1 h) with a secondary goat anti-mouse  $\mu$  chain antibody conjugated to alkaline phosphatase (Sigma-Aldrich, St. Louis, MO; catalog no. A7784), diluted 1:10,000 in 1% hemoglobin/TBST. Plates were washed with TBST four times and then incubated with the alkaline phosphatase substrate *p*-nitrophenyl phosphate (PNPP) (1 mg/ml in buffer 0.2 M Tris, pH 9.5, plus 100 mM NaCl plus 5 mM MgCl<sub>2</sub>) at RT for 1 h. Color intensity was determined with an enzyme-linked immunosorbent assay (ELISA) reader at an optical density at 405 nm (OD<sub>405</sub>).

**Periodate oxidation of the fungal cell surface carbohydrates.** Conidia of AF293 (2  $\times$  10<sup>6</sup>/ml) were seeded in 96-well plates with RPMI/MOPS and grown overnight at 37°C. On the following day, the plates were washed, and sodium meta-periodate (50 mM) was added to the AF293 hyphae and placed in the dark at 4°C and incubated overnight as described previously (32). Control plates were incubated with PBS. Plates were then washed three times with PBS followed by incubation with glycine buffer (250 mM, pH 7.8) for 1 h at RT in the dark. Plates

were then washed an additional three times with PBS and blocked with 1% hemoglobin in TBST buffer (20 mM Tris, pH 8.0; 140 mM NaCl; 0.05% Tween 20) for 1 h at 37°C. The binding of the anti-*A. fumigatus* MAb MPS5.44 to the periodate-treated *A. fumigatus* was determined, as described above.

**Fungicidal properties of the MAb-alliinase conjugate.** The antifungal activity of MAb (MPS5.44)-alliinase conjugates was determined according to the conditions of CLSI document M38-A2 (7). Resting conidia (3  $\times$  10<sup>4</sup> conidia/well) were seeded in 96-well plates and incubated for 4 h at 37°C with RPMI/MOPS (100  $\mu$ l). Conjugate MAb-alliinase was applied in serial twofold dilutions in triplicate, incubated for 30 min at 37°C, and then washed four times, and the addition of alliin (0.5 mg/ml) followed. Hyphal growth was monitored by microscopic observation as well as by OD<sub>595</sub> or by CBS/DsRed fluorescence (excitation at 540 nm, emission at 595 nm). MIC readings were initially taken after 24 h of incubation with alliin; the plates were then reexamined after 48 and 72 h. No changes in the MICs were noted after these periods. The wells in which no fungal germination was observed were scraped and plated on Sabouraud dextrose agar plates. The number of colonies was counted after 72 h to determine the minimal fungicidal concentration (MFC). As a control, the antifungal activity was also determined by incubating *A. fumigatus* with nonconjugated alliinase, as described above, or with conjugates consisting of the nonspecific MAb and alliinase.

**Pulmonary challenge and determination of fungal infection in mice.** Eight-week-old ICR female mice (25 to 28 g [body weight]) were maintained under specific pathogen-free conditions and handled according to protocols approved by the Weizmann Institute's Animal Care and Use Committee and adhering to international guidelines. Mice were immunosuppressed and challenged as previously described (54). Briefly, cortisone (25 mg in 200  $\mu$ l PBS) was injected intraperitoneally (i.p.) on days 0 and 3. Prior to infection, mice were anesthetized with isoflurane, and 10<sup>7</sup> DsRed conidia in 50  $\mu$ l PBS were inoculated intranasally on day 0.

In a preliminary experiment, we determined the optimal volumes as well as the dosages of the conjugates and alliin that could be introduced by i.t. instillation without causing animal discomfort. We found that i.t. administration of 50 nmol conjugate in 50  $\mu$ l PBS followed 30 min later by 750  $\mu$ g alliin in 25  $\mu$ l PBS was well tolerated by the mice. We then carried out a short-term study of tissue burden. For this experiment two groups of mice ( $n = 5$  each) were infected, as described above. One hour after infection, the animals in the control group were mock treated by i.t. instillation with PBS (50  $\mu$ l). The second group received a solution of MAb-alliinase conjugate (50 nmol conjugate in 50  $\mu$ l PBS), administered i.t. Thirty minutes later, alliin (750  $\mu$ g in 25  $\mu$ l PBS) was administered i.t. to both groups. All the infected animals survived the i.t. administrations. On day 4, the animals were euthanized, and the infection readout was carried out on fresh sections of inflated lungs using a confocal microscope. Fungal burden was determined in aliquots (20 and 100  $\mu$ l) of the lung tissue homogenate in PBS (total volume of 2 ml) by CFU enumeration on Sabouraud dextrose agar plates and extrapolated to the whole lung.

**Animal survival experiments.** Animals infected as described above were divided into groups ( $n = 15$ ). One hour after infection, animals in group G-1 (placebo) were treated i.t. with PBS (50  $\mu$ l), which was followed 30 min later by the administration of PBS (25  $\mu$ l). Animals in group G-2 were treated with PBS (50  $\mu$ l), which was followed by the administration of alliin (750  $\mu$ g/25  $\mu$ l PBS). Other groups were treated as follows: group G-3 with unconjugated MAb MPS5.44 (50 nmol/50  $\mu$ l PBS) followed by the administration of PBS (25  $\mu$ l); group G-4, with MAb-alliinase conjugate (50 nmol/50  $\mu$ l PBS) followed by the administration of PBS (25  $\mu$ l); group G-5 with MAb-alliinase conjugate (50 nmol/50  $\mu$ l PBS) followed by i.t. administration of alliin (750  $\mu$ g/25  $\mu$ l PBS); and group G-6 with unconjugated alliinase (50 nmol/50  $\mu$ l PBS) followed by alliin (750  $\mu$ g/25  $\mu$ l PBS). For group G-7 (treatment of fulminant IA), i.t. administration of conjugate (50 nmol/50  $\mu$ l PBS) and alliin (750  $\mu$ g/25  $\mu$ l PBS) was delayed for 50 h postinfection. As an antifungal drug control, group G-8 animals ( $n = 10$ ) were treated by i.t. with amphotericin B (AMB) (1 mg/kg) 1 h postinfection. All treatments were repeated on days 4, 6, and 9 postinfection, and animal survival in the different groups was followed for 36 days.

Evaluation of fungal infection in the lungs of mice that survived and were euthanized after 36 days or those that died during the survival experiment was performed in two ways. One lobe was fixed in 4% PBS-buffered formalin, embedded in paraffin, and cut into 5- $\mu$ m thick sections. For fungal detection, sections were stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) and examined microscopically. The remaining four lobes were homogenized in 2 ml PBS, and aliquots were seeded on Sabouraud dextrose agar plates for CFU enumeration of fungal burden.

**Statistical analysis.** Survival data were analyzed by the Kaplan-Meier method using GraphPad Prism 5 software (GraphPad Inc.). Differences in survival curves were assessed by the log rank test. Data from CFU counts from the lungs were

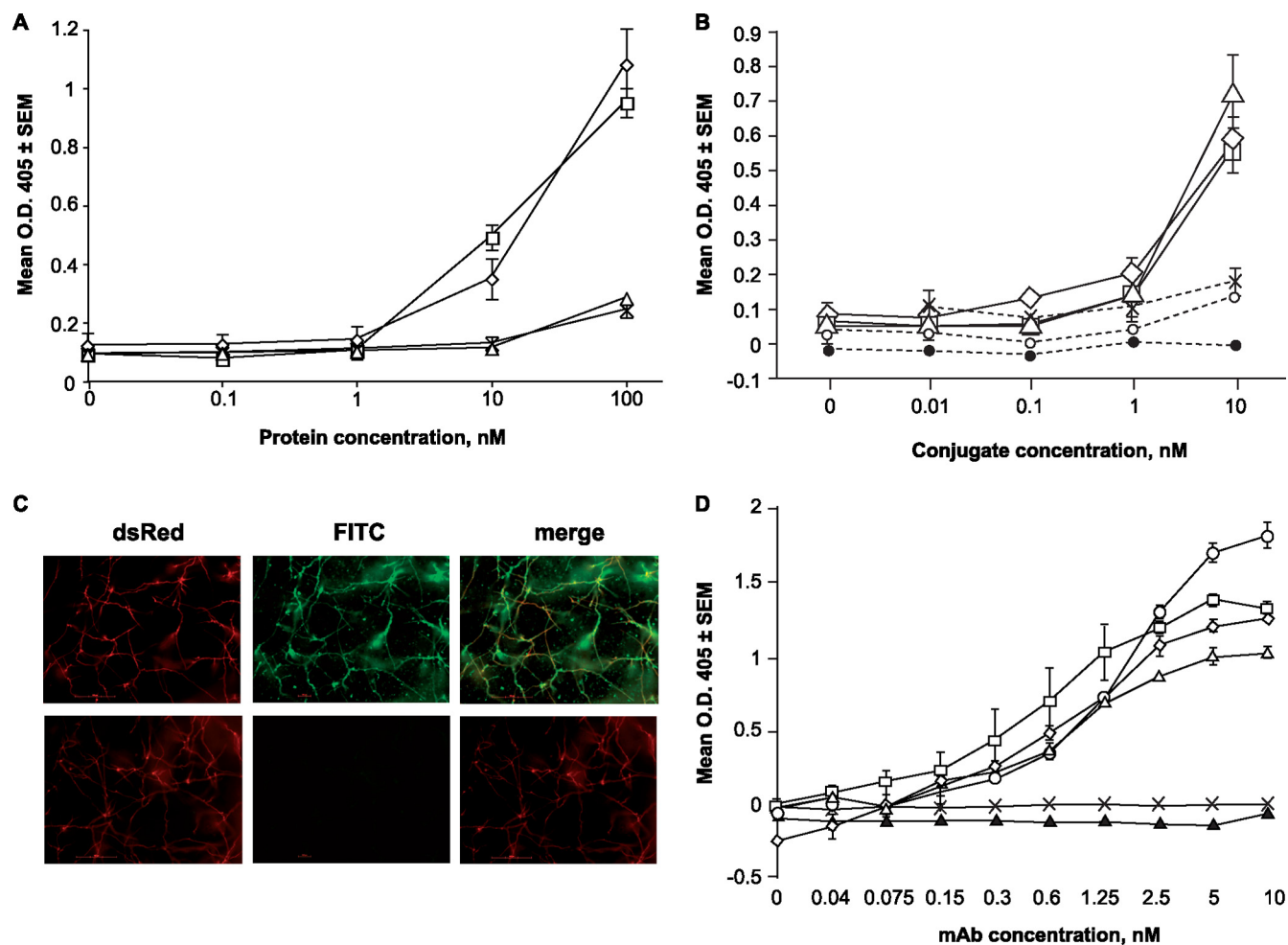


FIG. 1. Binding of the antibody and conjugates to *A. fumigatus*. Serial dilutions of the conjugates, MPS5.44 antibodies, or nonspecific antibody were applied on hyphae or conidia, and their binding was determined as described in Materials and Methods. (A) ELISA binding curves to hyphae of *A. fumigatus*. ◇, conjugate MAb (MPS5.44)-alliinase; □, antibody MPS5.44; ×, nonspecific mouse IgM; △, conjugate of nonspecific IgM with alliinase. (B) Conjugate MAb (MPS5.44)-alliinase bound efficiently to all forms of *A. fumigatus*, including swollen conidia (□) and resting conidia (◇), as well as hyphae (△). Binding of a nonspecific mouse IgM-alliinase conjugate (dashed lines) to all forms of *A. fumigatus* was negligible. ×, swollen; ○, resting conidia, and ●, hyphae. (C) Comparison between the binding of FITC-labeled conjugate (top row) and FITC-labeled alliinase (bottom row) to hyphae of *A. fumigatus* CBS 144.89/DsRed. FITC-labeling of the proteins was carried out according to manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). (D) Species specificity of MAb MPS 5.44. Binding curves of MAb to hyphae of different fungi as determined by ELISA. The binding of the MAb is expressed as mean OD<sub>405</sub> value readings from triplicate wells after subtraction of the OD value from the wells with the nonspecific MAb. ◇, *A. fumigatus* 293; □, *A. flavus*; ○, *A. niger*; △, *A. terreus* 9; ▲, *C. krusei*; \*, *C. albicans*.

analyzed by the unpaired two-tailed *t* test with Welch's correction and by the nonparametric two-tailed Mann-Whitney U test. For statistical significance, ELISA data were assessed by curve fit analysis.

## RESULTS

**Binding properties of the MAb (MPS5.44)-alliinase conjugate.** We first compared the binding of the *Aspergillus* cell wall-specific nonconjugated MAb (MPS5.44) to that of the MAb (MPS5.44)-alliinase conjugates. We found that both the free MAb and the conjugate bound the hyphae of either of the two *A. fumigatus* strains at similar concentrations (1 to 10 nM) (Fig. 1A). Curve fit analysis indicated no statistically significant difference between the binding curves of the conjugate and the free MAb ( $P > 0.05$ ). Notably, at these concentrations, no significant binding was detected with the nonspecific IgM anti-

body used as a control or with the conjugate prepared with the nonspecific MAb (Fig. 1A). Conjugates of MAb (MPS5.44)-alliinase bound all *A. fumigatus* forms, i.e., resting or swollen conidia as well as hyphae at similar concentrations (Fig. 1B).

Fluorescence microscopy was used to compare the binding of an FITC-labeled conjugate to the dsRed-*A. fumigatus* hyphae with that of FITC-labeled free alliinase (Fig. 1C). The results show that the binding of the conjugate to the red fluorescent hyphae (dsRed) was visible (Fig. 1C, top row), whereas binding of free, nonconjugated alliinase (Fig. 1C, bottom row) was almost undetectable. This finding demonstrated that the nonspecific adherence of the unconjugated alliinase to hyphae was much weaker than the binding of MAb MPS5.44-alliinase conjugate and that the anchoring of the conjugate was due to the specificity of the antifungal MAb. Furthermore, the



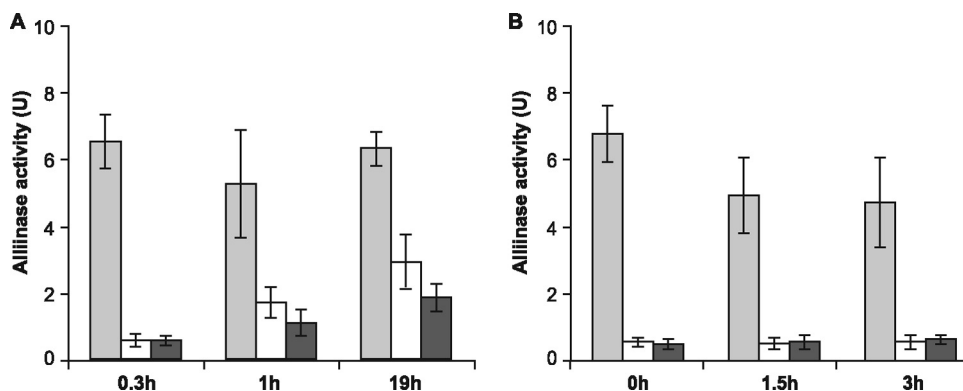


FIG. 2. Alliinase activity of the conjugate bound to *A. fumigatus*. (A) Saturation of the conidia with (i) MPS5.44-alliinase conjugates (light gray columns), (ii) a nonspecific IgM-alliinase conjugate (empty columns), or (iii) free alliinase (dark columns). Hyphae or swollen conidia were incubated, in triplicate, with 50 nmol conjugate or control proteins for various periods (20 min, 1 h, or 19 h). In each case the alliinase activity was determined immediately after removal of the enzyme. The results show the level of alliinase activity that remained anchored on the conidia at the indicated time. (B) Fifty nmol of MAb-alliinase conjugate or nonconjugated alliinase (as described in panel A) were incubated (30 min) with conidia. After washing of the unbound conjugate or enzyme, the level of alliinase activity that remained anchored on the fungus was determined at the indicated times.

control conjugate containing the nonspecific IgM MAb did not adhere to the hyphae (not shown). Specific binding of the conjugate to either *A. fumigatus* conidia or hyphae was rapid and reached saturation in 20 min. Further incubation for 1 h or longer did not increase the binding of the conjugate (Fig. 2A).

Importantly, the alliinase activity of the conjugates was preserved on the surface of conidia for at least 3 h (Fig. 2B), suggesting that the conjugate did not undergo clearance from the fungal surface and the enzymatic activity of alliinase remained at a similar level. The alliinase activity of the bound MAb (MPS5.44)-alliinase conjugate was significantly higher ( $P < 0.001$ ) than the alliinase activity of the conjugate with the nonspecific MAb or of the unconjugated alliinase (Fig. 2B). In addition, pretreatment of *A. fumigatus* hyphae with sodium *meta*-periodate, which degrades sugar molecules containing vicinal hydroxyl groups, completely abrogated the binding of MAb MPS5.44 (Fig. 3), indicating that the antibody recognizes a polysaccharide on the surface of the fungus. Notably, the anti-*A. fumigatus* MAb MPS5.44 bound other pathogenic *Aspergillus* species, such as *A. niger*, *A. flavus*, and *A. terreus*, with

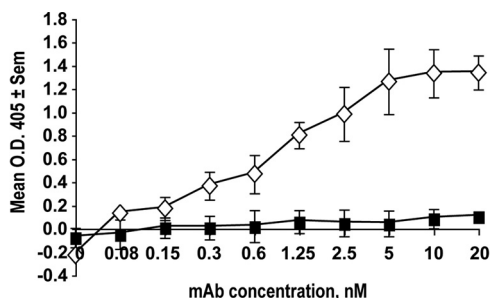


FIG. 3. Effect of periodate oxidation of *A. fumigatus* hyphae on the binding of MAb MPS5.44. ELISA binding curves of MAb to hyphae of *A. fumigatus* 293 pretreated with sodium *meta*-periodate (■) or non-treated (◇) were determined as described in Materials and Methods. Binding is expressed as mean spectrophotometric readings ( $OD_{405}$ ) from triplicate wells after subtraction of OD values from the wells with nonspecific MAb. Mean  $\pm$  standard error of the mean (SEM) values are shown.

similar high affinities, suggesting that it interacts with a shared *Aspergillus* surface carbohydrate epitope (Fig. 1D). In contrast, no binding to either *C. albicans* or *C. krusei* (Fig. 1D) or to a *Mucor* mold was observed (data not shown).

**In vitro fungicidal properties of the conjugates.** The antifungal activity of the conjugate was determined on swollen conidia which were preincubated (30 min, 37°C) with serial dilutions of conjugate; the unbound conjugate was washed, and alliin was added. The MIC was estimated at 1.25 to 2.5 nM. Complete sterility (MFC) was achieved with conjugate concentrations as low as 5 to 10 nM. Curve fit analysis indicated statistically significant ( $P < 0.01$ ) differences between CFU counts determined after treatment with the MAb-alliinase conjugate and alliin and those after treatment with the nonspecific conjugate or with free alliinase and alliin (Fig. 4A). The MFC for resting conidia treated likewise was similar, whereas for hyphae it was 25 nM (Fig. 4B). This higher value may reflect a difference in fungal mass. In comparison, the MFC of pure alliin was 7.5  $\mu$ g/ml for swollen conidia, 15  $\mu$ g/ml for resting conidia, and 30  $\mu$ g/ml for hyphae (data not shown). The MIC was two- to fourfold lower than the MFC in all cases.

Swollen conidia preincubated with unconjugated alliinase or with the nonspecific MAb-alliinase conjugate, to which alliin was added, were not killed, even at the highest concentration examined (50 nmol) (Fig. 4A and B). Notably, neither MAb MPS5.44 alone nor conjugate without alliin inhibited germination of conidia or hyphae formation when applied for short periods (30 min followed by washing) (Fig. 4A). Prolonged incubations (19 h) of conidia with unconjugated MAb MPS5.44 (20 nmol) resulted in partial inhibition of hyphal formation (Fig. 4C, panels C1 and C2). Conjugate applied for the same period but without alliin also had some inhibitory effect (Fig. 4C, panel C4). Upon addition of alliin (0.5 mg/ml), the concentrations of the conjugate could be decreased to as low as 10 pmol for a similar suppression of fungal growth (Fig. 4C, panel C3). In conclusion, both the antibody alone and the conjugate without alliin had low anti-*A. fumigatus* activity. In the presence of alliin, the effective fungicidal concentrations of the conjugate were three orders of magnitude lower.

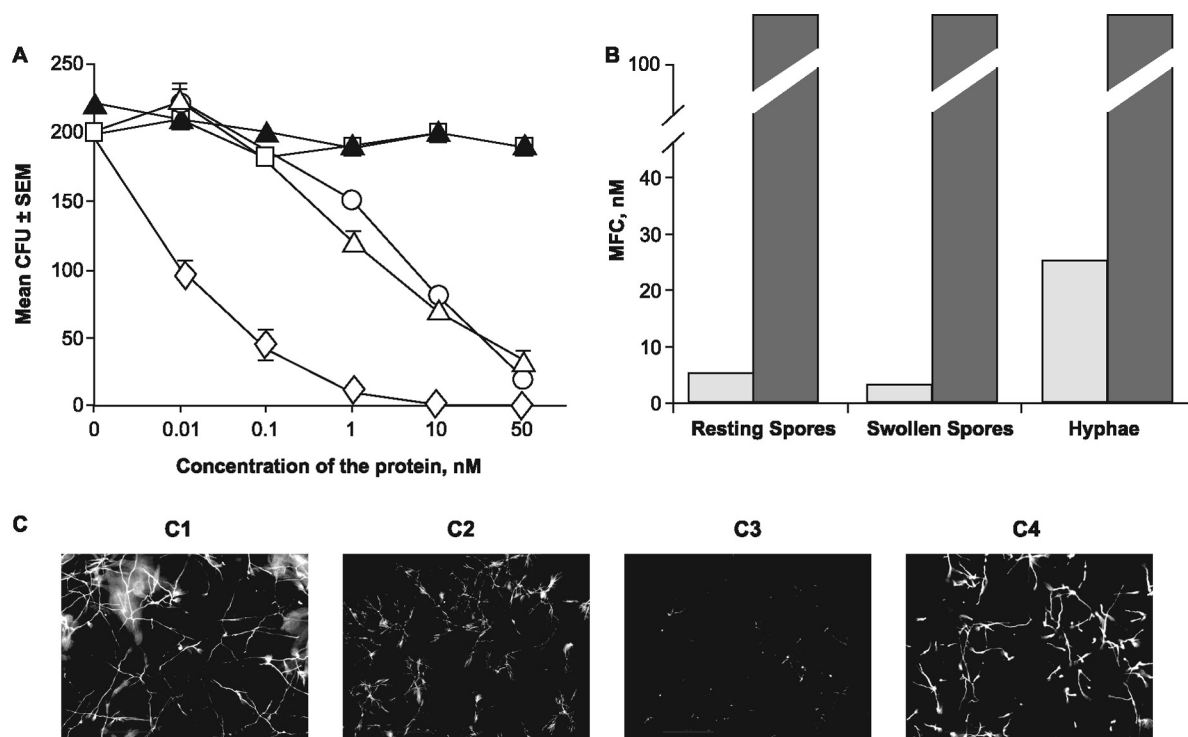


FIG. 4. Fungicidal activity of the conjugates in the presence of alliin. (A) Inhibition of fungal growth by the MAB-alliinase conjugate, the unconjugated MAB, or the nonconjugated alliinase. The conjugated and unconjugated MABs were applied on swollen conidia (in triplicate) at the indicated concentrations for 30 min. Plates were washed, and fungi were allowed to grow (72 h, 37°C) in the presence or absence of alliin (0.5 mg/ml). Wells in which no germination was observed were scraped and seeded on Sabouraud dextrose agar plates, and the number of CFU were counted 72 h later. ◇, MAB (MPS5.44)-alliinase conjugate and alliin; □, MAB (MPS5.44)-alliinase conjugate without alliin; ▲, unconjugated MAB (MPS5.44); ○, nonconjugated alliinase and alliin; △, conjugate of nonspecific MAB IgM-alliinase and alliin. (B) MFC of the MAB (MPS5.44)-alliinase conjugate (light gray columns) or of unconjugated alliinase (dark columns) for resting conidia, swollen conidia, and hyphae. Conidia or hyphae were incubated with serial dilutions of conjugate or alliinase for 30 min, and alliin was added after removal of unbound proteins, as described in panel A. (C) Hyphal growth (19 h, 37°C). C1, control, in the absence of antibody; C2, in the presence of 20 nmol unconjugated MAB MPS5.44; C3, 10 pmol of MAB (MPS5.44)-alliinase conjugate with alliin (0.5 mg/ml); C4, 20 nmol MAB (MPS5.44)-alliinase conjugate without alliin. Scale bar, 100  $\mu$ m.

**Conjugate and alliin treatment significantly reduces lung fungal load in *A. fumigatus*-infected mice.** In order to estimate the short-term development of pulmonary fungal infection and to test the effects of i.t. administration of conjugate and alliin at the early stage, two groups of immunosuppressed mice ( $n = 5$ ) were infected with DsRed conidia. The optimal number of DsRed conidia required to obtain a reproducible rate of infection in mice was previously established (54). The mice were euthanized on day 4, and the five lobes of their lungs were sliced and examined by confocal microscope to determine the number of fluorescent fungal colonies. Animals in the control group that were treated with PBS and alliin but did not receive conjugate had large numbers ( $290 \pm 56$ ) of fungal colonies throughout their lungs. One representative colony is shown in Fig. 5A. In comparison, mice treated with the conjugate and alliin had very few visible small colonies ( $28 \pm 9.4$ ) in their lungs, significantly lower ( $P < 0.01$ ) than the control group (Fig. 5B). Fungal burden was also determined in homogenates of lung lobes, revealing a high number of CFU per lung ( $442 \pm 78$ ) in the PBS-treated group versus  $3.3 \pm 3.3$  CFU in the conjugate-treated group ( $P < 0.01$ ), which correlated well with the above-described microscopic enumeration. In conclusion, on day 4 postchallenge, mice treated with placebo had numer-

ous hyphal colonies spread throughout their lungs, whereas animals that received a single i.t. treatment of conjugate and alliin had almost no *A. fumigatus*.

**Conjugate and alliin treatment significantly reduced mortality in *A. fumigatus*-infected mice.** In order to assess the effect of treatment with conjugate and alliin on long-term survival (36 days) of *A. fumigatus*-infected mice, an additional experiment was carried out with larger groups of animals ( $n = 15$ ) as described in Materials and Methods. As shown in Fig. 6, 100% of the *A. fumigatus*-infected mice in the control groups that received PBS instead of conjugate (G-1 and G-2) died by day 13 (median survival time [MST] = 7 days). Infected mice treated with unconjugated MABs (G-3) or conjugate without alliin (G-4) exhibited 80% mortality at 36 days (MST = 18 days). The log rank test indicated a statistically significant difference between the survival curves of placebo-treated animals and those treated with either unconjugated MAB or conjugate without alliin ( $P < 0.001$ ). The i.t. administration of unconjugated MAB MPS5.44 (G-3) had some protective effect, as it increased the median survival time of the infected mice by 11 days (Fig. 6). Importantly, the most impressive therapeutic effect (>85% survival) was seen with G-5 mice that were treated with conjugate and alliin. Two of these mice died not

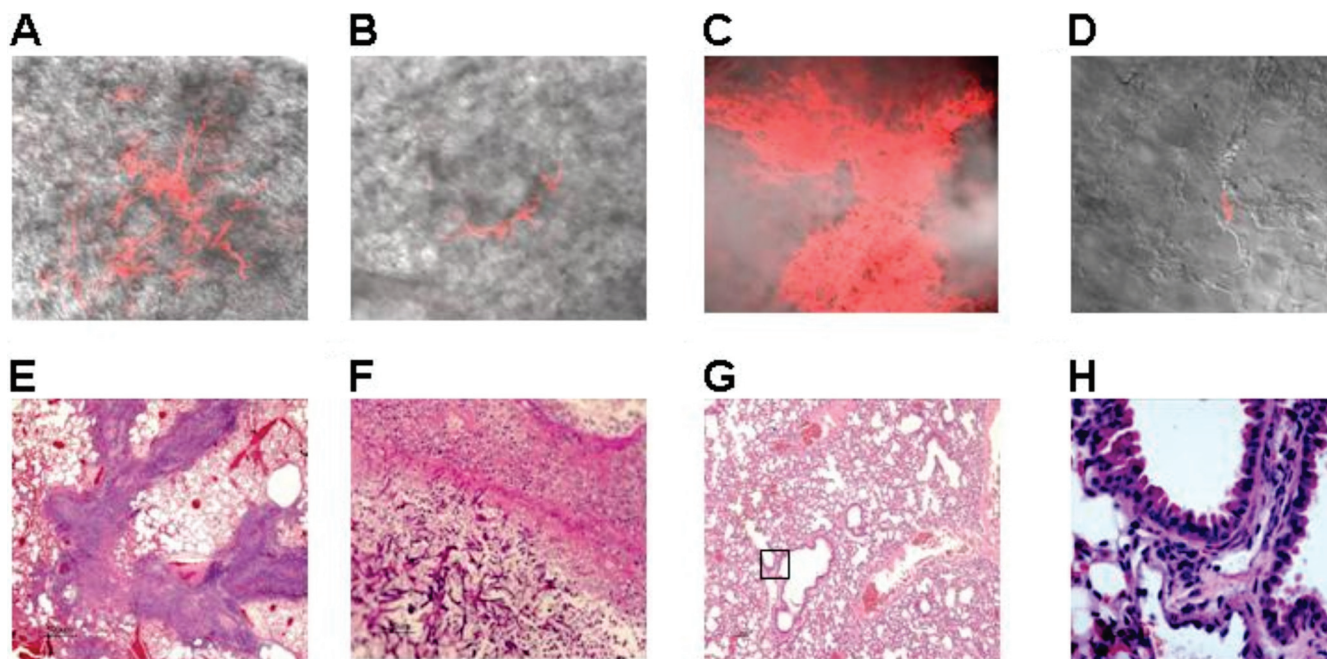


FIG. 5. Detection of fungal infection in lung tissues. Confocal microscopic analysis of the lungs of PBS-treated control (A and C) or MAb-alliinase-conjugate-plus-alliin-treated mice (B and D). Infection readout was carried out on fresh tissue sections isolated from inflated lungs, using a Zeiss Axioplan confocal microscope and LSM 510 software. *A. fumigatus* formed red fluorescent colonies in lungs at day 4 after infection. A representative picture (from one mouse out of five) of PBS-and-alliin-treated mice (A); a representative field (one out of five) of animals treated with MAb-alliinase conjugate and alliin (B); a representative picture of a PBS-treated mouse taken at a later time point (day 13) by which time the fungi had spread throughout the lungs (C); a representative picture showing tiny colonies, if any, in lungs of a mouse treated four times with conjugate and alliin (euthanized on day 36) (D). (E to H) Histological examination of lung sections of infected mice. Paraffin sections of the lungs were stained with H&E (E, G, and H) and PAS (F). Severe necrotizing bronchitis was seen with placebo-treated mice, with invasion of vascular walls by fungal hyphae. Panels E and F show representative lung sections from an animal who died during the second week of infection. Panels G and H show representative lung sections from mice of groups G-5 and G-7 that were treated four times with MAb-alliinase conjugate and alliin and were euthanized on day 36. Their lungs were found to have normal airway epithelium. Magnification,  $\times 20$  (panels A to D),  $\times 10$  (panels E and G), and  $\times 40$  (panels F and H).

long after infection (days 7 and 11), but the rest survived for the duration of the experiment (36 days).

To determine whether the efficacy of the conjugate-based treatment was antibody dependent, animals in G-6 were treated with unconjugated alliinase and alliin (Fig. 6). The

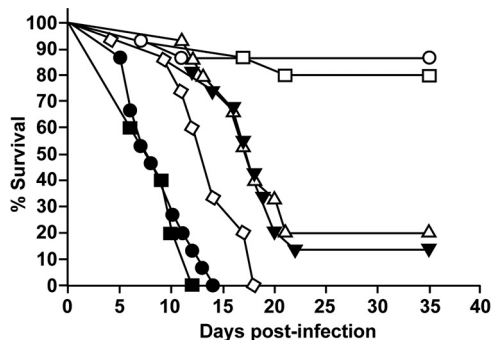


FIG. 6. Survival of infected mice. ●, group G-1 (control), PBS-treated mice; ■, G-2, PBS plus alliin; △, G-3, unconjugated MAB (MPS 5.44) plus PBS; ▼, G-4, MAB-alliinase conjugate with PBS (no alliin); ○, G-5, mice treated with MAb-alliinase conjugate and alliin (treatment started on day 0); ◇, G-6, nonconjugated alliinase and alliin; □, G-7, MAB-alliinase conjugate and alliin (treatment started on day 2).

alliinase concentration (50 nmol/50  $\mu$ l) and its specific activity (120 U/nmol) were similar to that of the alliinase that was ligated in the conjugates. Following this treatment, G-6 mice began dying on day 4 postinfection; none survived for over 3 weeks (MST = 14 days) (Fig. 6). The survival curves of the animals treated with nonconjugated alliinase and alliin (G-6) significantly differed from (i) control (PBS and alliin)-treated mice (G-2) ( $P < 0.01$ ) as well as from (ii) the conjugate-and-alliin-treated animals (G-5) ( $P < 0.001$ ). This result indicates that alliin produced in the bronchial space by the unconjugated alliinase had some antifungal activity but was not as effective as that produced by the MAb-targeted alliinase anchored to the surface of the fungus (G-5).

We also tested whether treatment with the conjugate and alliin could have a therapeutic effect on a more advanced pulmonary infection. For this purpose we delayed the intratracheal administration of conjugate and alliin in the infected G-7 mice only, commencing treatment 50 h postinfection. Treatment was repeated on days 4, 6, and 9. As can be seen in Fig. 6, 80% of the mice in this group survived for the duration of the experiment (36 days), a rate comparable to that of G-5 ( $P > 0.05$ ), in which the treatments with conjugate and alliin, as mentioned above, coincided with the day of infection. Thus, treatments with conjugate and alliin protected the majority of animals ( $>80\%$ ), even when begun at a more advanced stage of infec-



tion. Notably, in both cases, treatment with conjugate and alliin was more effective than the gold-standard treatment with the antifungal drug AMB (G-8, MST = 18 days), in which only 30% of mice survived at the end of the experiment.

Lung tissues from placebo (G-1 and G-2)- and conjugate (G-5 and G-7)-treated animals were examined by histology and fungal burden analysis for the presence of *A. fumigatus*. The results indicate a high fungal burden ( $6,700 \pm 2,056$  colonies per lung) and extensive invasive pulmonary aspergillosis in the lungs of placebo-treated mice (Fig. 5C, E, and F) compared to a very low fungal burden ( $4.3 \pm 2.3$  colonies per lung;  $P < 0.01$ ) and no apparent fungal growth in the lungs of conjugate/alliin-treated mice euthanized at the end of the experiment (Fig. 5D, G, and H). Furthermore, lung tissue of conjugate-and-alliin-treated mice appeared to be intact; no damage was observed by microscopical examination of hematoxylin-and-eosin-stained lung sections due to the allicin produced *in situ* (Fig. 5G and H). In addition, no tissue damage was observed with either the liver or kidneys of the conjugate-and-alliin-treated mice that survived (data not shown).

## DISCUSSION

Invasive aspergillosis has become an increasing cause of morbidity and mortality in immunocompromised individuals, those with AIDS as well as those undergoing allogeneic bone marrow transplantations or intensive chemotherapy (10, 25, 48). In many cases, infection is due to the inhalation of conidia of *A. fumigatus*, which germinate and grow in the small airways and alveolar spaces of the lung (22, 55). Studies of *postmortem* lung specimens have shown that *A. fumigatus* is present and viable more frequently than would be expected from its prevalence among the fungal conidia normally found in the air (33). In healthy individuals, a number of shared defense mechanisms are activated in response to a range of fungi. Neutrophils, alveolar macrophages, and monocyte effector cells have a fundamental antifungal function (40, 47). Resident and monocyte-derived macrophages ingest and kill *Aspergillus* conidia, preventing their transition to the invasive hyphal form (5, 18, 42, 43). Treatment with high doses of corticosteroids, which suppress neutrophil and macrophage function, predisposes patients to IPA (24, 42). In many such cases, bronchoalveolar macrophages fail to control the fungi (55), and conidia germinate into hyphae, pierce the thin alveolar barrier, and invade the underlying blood vessels.

Numerous antifungal strategies to treat IPA infections have been reported. Most are based on azoles, such as voriconazole (52), or on formulations and applications of AMB (6, 31, 41) or echinocandins (12, 16, 23). Recent studies of animal models have demonstrated the therapeutic potential of monoclonal antibodies (9, 53) as well as of ultrashort synthetic lipopeptides (54) with several types of fungal infections. Another report indicated that anti-idiotypic antibodies administered intranasally could effectively control *A. fumigatus* infection in neutropenic mice (8). Furthermore, a combination of an antifungal HSP90 antibody with AMB improved recovery in patients with invasive candidiasis (26).

The intranasal infection of immunosuppressed mice with conidia of *A. fumigatus* has been shown to be a suitable model of invasive pulmonary aspergillosis, which leads to animal

death within 7 to 12 days (54). In this model, corticosteroids were administered to the mice to inhibit the killing of phagocytosed fungi and to suppress the activity of neutrophils. The main aim of our investigation was to test *in vivo* the antifungal effect of allicin when produced at the *A. fumigatus* surface target. For this purpose we produced an anti-*A. fumigatus* MAb that was selected due to its capacity to specifically bind to the *A. fumigatus* cell surfaces. The *A. fumigatus* MAb was then chemically ligated to the alliinase enzyme which catalyzes the production of allicin from the natural substrate alliin (39). To establish a proof of principle for treating murine invasive pulmonary aspergillosis with the MAb-alliinase conjugate and alliin, we chose the intratracheal route of administration (17) for both the conjugate and the alliin. Intratracheal treatment has been shown to provide in some cases an increased therapeutic benefit (34, 49) in comparison to parenteral or other nonparenteral routes. Furthermore, since the pulmonary infection is predominant in cases of aspergillosis, i.t. treatment appeared to be the most convenient way to deliver the drug to the targeted pathogen.

In the present study, four i.t. administrations of the conjugate and alliin resulted in 80% to 86% total recovery of the infected mice, with almost complete fungal clearance. No tissue damage by the allicin produced *in situ* was observed with the surviving animals. Treatment with the conjugate and alliin was successful in both (i) a system analogous to prophylactic therapy, in which administration of the conjugate and alliin started at day 0, 1 h after the intranasal inhalation of conidia, and (ii) a situation in which the disease had already spread (50 h postinfection) and hyphae had begun to emerge in the lung tissue. In both instances, the four repetitive treatments with conjugate and alliin were able to destroy the fungi, and over 80% of the infected animals recovered with complete fungal clearance, as revealed by histology and fungal burden analysis. In contrast, in all our control experiments in which we used either unconjugated MAb, conjugate alone (without alliin), nonconjugated alliinase and alliin, or PBS and alliin, the infection was very extensive and most of the mice died in less than 20 days. These results clearly indicate that anchoring the alliinase enzyme to the surface of the fungi by the anti-*Aspergillus* antibody enabled the targeted production of allicin, which was crucial for the efficacy of the antifungal treatment.

It is noteworthy that the new monoclonal antibody against *A. fumigatus*, MAb MPS5.44, was found to have in itself some protective effect, as had previously been reported for other monoclonal antibodies (9). Our MAb was also found to cross-react with other *Aspergillus* species, such as *A. niger*, *A. flavus*, and *A. terreus*, though it did not recognize *C. albicans*, *C. krusei*, or a *Mucor* mold. Thus, it is quite possible that the present MAb-alliinase conjugates could be useful against infections caused by other aspergilli; this hypothesis will be investigated in the future.

In conclusion, our results demonstrate that it is possible to target the production of cytotoxic molecules of allicin against *A. fumigatus*. The present study is thought to be the first known example of the successful use of the antibody-directed enzyme prodrug technology (ADEPT) (4) for the treatment of a potentially fatal fungal infection.

The limitations of this study are, however, that the demonstration was achieved in a constrained animal setting in which

the conjugate and alliin were administered directly into the infected lung. A comparison between the intratracheal route of delivery of the MAB-alliinase conjugate and alliin and other parenteral and nonparenteral routes for the separate delivery of the drug components in models of pulmonary and extrapulmonary aspergillosis will be undertaken.

One important advantage of the MAB-alliinase approach is that the prodrug, alliin, is a water soluble, nontoxic natural compound which has been declared to be a GRAS substance by the FDA. Upon its interaction with alliinase, the hydrophilic alliin substrate is converted into hydrophobic allicin molecules which rapidly permeate the fungal cell membrane and react with free thiol groups which are present on numerous cellular components (2, 29, 36), causing death to the targeted pathogen. Our results thus far suggest that the introduction of antifungal conjugates that can produce targeted allicin molecules may treat IPA more effectively than the current therapies, although this will have to be confirmed, as mentioned above, by investigations with additional models of aspergillosis. The targeted principle, however, could also be used for the development of additional antimicrobial applications.

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The Weizmann Institute has submitted on behalf of the authors E.A., A.R., and D.M. a patent application regarding the MAB (MPS5.44)-alliinase conjugate.

The authors declare that they have no conflict of interest with respect to this work.

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