Aerosolized sodium hypochlorite inhibits viability and allergenicity of mold on building materials

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Background: Commercial and residential buildings can become contaminated with molds, which may trigger allergic disorders. Mold remediation efforts may require costly replacement of mold-contaminated building materials. Disinfectants that contain dilute sodium hypochlorite can kill mold and are practical to use. Whether they also inhibit mold allergy symptoms is unknown.

Objective: We tested the hypothesis that sodium hypochloritecontaining spray products kill *Aspergillus fumigatus* and inhibit *A fumigatus* allergens.

Methods: A fumigatus was grown on 3 common building construction materials, as well as in solution by conventional laboratory methods. Two sodium hypochlorite-containing household products (diluted bleach and Tilex) were sprayed on the mold-contaminated materials or added to mold in solution and compared with untreated controls. Surface mold and associated debris were mechanically removed from treated and untreated boards. Conidia in the extracted board materials were quantified by light microscopy, examined for morphologic changes by scanning electron microscopy, and cultured for viable mold. Extracts were tested for A fumigatus antigen by ELISA, and for A fumigatus allergen by skin prick testing using extracts prepared from both the boards and the cultured solutions.

Results: Both sodium hypochlorite disinfectants killed *A fumigatus* in solution and on mold-contaminated building materials. Light microscopy and scanning electron microscopy demonstrated changes to the conidial surface. Both dilute bleach and Tilex inhibited *A fumigatus* recognition by ELISA. Skin testing supported the results of the ELISAs and demonstrated loss of skin test reactivity to the sodium hypochlorite–treated mold solutions in most of the subjects. Of the 4 individuals who had a positive skin test result to mold grown on oriented strand board building material, 3 no longer reacted to extracts from bleach-treated boards. Conclusion: Spray application of sodium hypochlorite–containing disinfectants onto mold-contaminated building material kills *A fumigatus*, modifies the surface characteristics

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of *A fumigatus* conidia, reduces recognition of *A fumigatus* mold by ELISA, and results in loss of skin test reactivity to the treated mold in individuals allergic to *A fumigatus*. (J Allergy Clin Immunol 2005;116:630-5.)

Key words: Mold, bleach, Tilex, Aspergillus fumigatus, allergy, skin testing, building materials

The ability of household bleach (sodium hypochlorite) to disinfect surfaces is well known. Dilutions of bleach have been used since the 1800s in various applications to disinfect hospitals, laboratories, schools, prisons, and homes. Its widespread use reflects the ability of bleach to kill a broad spectrum of microorganisms in a concentration that presents little toxicity to individuals.

Household bleach typically consists of an aqueous solution of sodium hypochlorite between 4% and 6%. For disinfection, the bleach concentration is diluted to 1:16 by adding $\frac{1}{4}$ to $\frac{1}{2}$ cups of bleach per gallon of water. The resulting solution contains about 5000 ppm of sodium hypochlorite,¹⁻⁵ which is sprayed or wiped onto the surface to be disinfected. Although the actual mode of action is unknown, the primary active agent is undissociated hypochlorous acid.¹

Although sodium hypochlorite can kill molds *in vitro*,¹ less is known about the ability of bleach to denature mold allergens. The United States Environmental Protection Agency indicates that because most health complaints associated with exposure to mold are allergic, simply killing the mold may not reduce symptoms.⁶ Other authors support this contention.⁵ As such, the EPA and other authorities do not recommend biocides to treat mold-contaminated surfaces, because dead mold may still be allergenic.^{6,7} However, to our knowledge, the ability of bleach to denature mold allergens has not been investigated.

Several studies have demonstrated that bleach can inactivate other household allergens. Chen and Eggleston⁸ showed that typical household concentrations of sodium hypochlorite fragmented mouse, cockroach, and dust mite proteins. In solution, sodium hypochlorite also reduced the detection of protein antigens by ELISA. Antigen levels also fell when allergen-contaminated smooth surfaces were wiped with sodium hypochlorite solution. More porous household surfaces and building materials were not tested. A similar study conducted by

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Abbreviations used CFU: Colony-forming unit OSB: Oriented strand board PBST: PBS containing 0.05% Tween 20 PDA: Potato dextrose agar

Matsui et al⁹ found that low concentrations of sodium hypochlorite reduced detection of cat antigen in solution. Together, these studies demonstrate the ability of sodium hypochlorite to reduce household allergens. Periera et al¹⁰ hypothesized that the mechanism for this effect was a result of chlorination of amide nitrogen followed by protein oxidation to an imine with further hydrolysis, thus denaturing allergenic proteins. To date, there are no published studies that show the inhibition of fungal allergens by sodium hypochlorite solution on environmental surfaces. This is a critical issue in light of the growing recognition of health hazards caused by mold contamination.⁵

To justify the use of a biocide such as sodium hypochlorite for the remediation of mold, the biocide should be effective in an environment heavily loaded with organic matter. It should also have an acceptable safety profile. The biocide should not only kill the mold but also reduce mold antigen load. The purpose of this project was to determine whether household bleach or a liquid sanitizer containing sodium hypochlorite could reduce both viable mold and detectable allergen on environmental surfaces.

METHODS

Growth of *Aspergillus fumigatus* and preparation of conidia

We obtained *Aspergillus fumigatus* from the American Type Culture Collection (#16913; Manasssas, Va) as a freeze-dried material. The fungus was reconstituted and inoculated onto potato dextrose agar (PDA) plates (Remel, Lenexa, Kan) and incubated at 30°C. Plates were inspected daily for growth and to ensure a pure culture. PDA slants were made of the initial pure culture and were stored at 4°C. The conidia were harvested by modification of the procedure described by Allen et al.¹¹ In brief, the conidia were harvested by gently scraping the fungal mat from a confluent culture of *A fumigatus* grown on PDA into sterile, distilled water. The number of conidia was determined by using a hemocytometer.¹¹

Effect of bleach in aqueous solution

The ability of bleach and Tilex (Clorox Co, Oakland, Calif) to kill *A fumigatus* was first determined by using a liquid culture of the organism. We prepared three 10-mL suspensions of the stock *A fumigatus* solution at 10^6 colony-forming units (CFU)/mL. An additional 10 mL sterile distilled water was added to 1 *A fumigatus* suspension as the control solution. A 10-mL solution of household strength sodium hypochlorite bleach (Clorox Co), prepared at 1 cup per gallon of water, or 1:16, was added to the second mold suspension. A 10-mL solution of undiluted Tilex containing 2.4% sodium hypochlorite plus surfactants was added to the third suspension. We used similar concentrations of bleach solutions to treat mold-contaminated building materials. All of the suspensions were

Building materials

We next cultivated *A fumigatus* on building materials that commonly grow mold after water intrusion. Three types of building materials were inoculated with conidia: (1) oriented strand board (OSB), (2) gypsum drywall, and (3) plywood. All 3 of these materials were purchased at a local lumberyard and cut into 9-cm by 9-cm squares. The squares were packaged into autoclave bags, autoclaved in steam heat for a period of 30 minutes, removed from the bags by using sterile technique in a biological safety cabinet, and placed into sterile Petri dishes 150 mm in diameter and 25 mm deep (Nalge Nunc, Naperville, III). Thirty milliliters of sterile, distilled water was added over the top of each of the building material squares. The plates were then covered and incubated at 30°C for a minimum of 24 hours before inoculation.

Inoculation of building materials

The plates were next divided into 6 treatment groups, with each condition performed in triplicate. Groups 1, 2, and 3 were inoculated with a 1-mL solution of $1 \times 10^5 A$ *fumigatus* conidia spread over the surface of the building material square by using a cell scraper. Groups 4 to 6 were control plates for the treatment conditions and not inoculated with mold.

All plates were further incubated for 14 days, at which time visible mold growth was evident on all of the mold-inoculated building material squares.

Treatment solutions on building materials

Groups 1 (with mold) and 4 (without mold) squares served as controls for the sodium hypochlorite treatment groups and were sprayed with 7.5 mL sterile water. Groups 2 (with mold) and 5 (without mold) building material squares were sprayed with a 1:16 solution of bleach to distilled water. To establish reliable and consistent application, each square was sprayed with 5 sprays at 1.5 mL/spray, for a total of 7.5 mL of each solution. Groups 3 (with mold) and 6 (without mold) were treated the same way with Tilex at the supplied commercial concentration. All building material squares were then incubated at 30°C for 24 hours. The mold on the building material squares (groups 1, 2, and 3) was removed by using a disposable cell lifter and placed into solutions of sterile distilled water. The negative control plates (groups 4, 5, and 6) were similarly scraped into solution and washed with sterile distilled water. All solutions were centrifuged and washed several times with sterile distilled water to stop any continuing action of the bleach and Tilex solutions.

Assessment of mold counts, culturability, and morphology

Mold culture. A portion of the solution obtained from each group of building material squares was serially diluted and plated on PDA plates to determine the culturable colony count (CFU) for each treatment.

Mold count. We counted the number of visible conidia in an undiluted aliquot of each treatment group by using a hemocytometer and a light microscope. We recorded the presence and extent of mold spore clumping and the presence of building material square debris for each of the treatment conditions.



FIG 1. Treated vs untreated *A fumigatus* conidia in solution. Final OD = raw absorbance minus absorbance of the blank at 540 nm. Results are mean and SEM of n = 11 experiments.

Morphology. Morphology of conidia was assessed qualitatively by using both light microscopy and scanning electron microscopy.

A fumigatus ELISA

Environmental and occupational respiratory disorders

The assay involved coating a 96-well microtiter plate (Dynex Immulon 2HB, Chantilly, Va) with 100 µL of an optimal concentration of goat anti-A fumigatus antibody (1/1000 or 1/2000) (Immuno-Mycologics, Norman, Okla) diluted in PBS. The optimal concentrations of all antibodies used in the ELISA were predetermined by using a checkerboard titration procedure. Plates were incubated overnight at 4°C, then washed 3 times with PBST. The wells were next incubated for 1 hour at 37°C with a blocking solution of 120 µL PBST containing 1% BSA. Standards, blanks, and treated suspensions in PBST-BSA were added to the wells in duplicate. The standard curve was based on 100-µL aliquots of A fumigatus conidia at of 10, 5, 2.5, 1.25, 0.63 and 0.31×10^{6} CFU/mL in PBST-BSA. After a 1-hour incubation at 37°C, plates were washed with PBST. Each well was then treated with 100 µL of an optimal concentration of rabbit anti-A fumigatus (1/1000 or 1/2000; Gibson Laboratories, Lexington, Ky) diluted in PBST-BSA, and incubated at 37°C for an additional 1 hour. After washing with PBST, 100 µL horseradish peroxidase-conjugated goat antirabbit IgG (Kirkegard & Perry, Gaithersburg, Md) in PBST was added to each well and incubated for an additional 30 minutes. After washing with PBST, 75 µL o-phenylenediamine dihydrochloride (Sigma, St Louis, Mo) at a concentration of 1.25 mg/mL distilled water with 20 μ L 30% H₂O₂ per 10 mL 3,3' dimethoxybenzidine was added to each well. The plate was incubated for 10 minutes at room temperature followed by the addition of 30 μ L 9 molar H₂SO₄. The plates were read at OD₅₄₀ nm on an ELISA plate reader. Results are reported as the mean of duplicate wells by optical density. The limit of detection for this ELISA is 0.63 to 3.2×10^{5} /mL conidia.

A fumigatus skin prick testing

After approval of a human research protocol by the National Jewish Institutional Review Board, we studied the effect of bleach and Tilex treatment on Aspergillus skin test extracts. We enrolled and screened 21 subjects who reported a clinical diagnosis of mold allergy. Past allergic status to A *fumigatus* was verified by review of previous skin testing records when available. After informed consent, study subjects withheld medications that could potentially interfere with skin test reactivity. To screen for adequate histamine and *Aspergillus* reactivity, subjects were skin prick tested to histamine, negative (saline) control, commercial *A fumigatus* extract

(Greer Laboratories, Lenoir, NC), and A fumigatus extract produced by National Jewish Clinical Immunology Laboratory with a modified commercial method by using acetone precipitation of the fungal antigens (Crenshaw R. Personal communication, October 2003). Allergen content of both extracts was determined by using Asp f 1 ELISA (Indoor Biotechnologies, Charlottesville, Va). Skin prick testing was performed by using Dualtip skin test applicators (Lincoln Diagnostic Inc, Decatur, Ill). Only subjects in whom A fumigatus skin test reactivity could be verified were included in the remainder of the study. These remaining subjects were skin prick tested to mold extracts prepared from solutions treated with distilled water, dilute (1:16) bleach, or undiluted Tilex. Mold extracts from treated and untreated OSB were also used. In addition, subjects were tested with extracts prepared from uninoculated OSB to ensure that they did not react to other components of board materials. Skin tests were measured by the same observer who was blinded to the solutions applied. The diameter of the wheal and flare response was recorded for each extract. A positive skin test result was one with a minimum wheal diameter of 3 mm and a minimum flare diameter of 10 mm.

Statistical methods

A paired comparison was made between treatment groups: (1) building squares inoculated with mold and treated with water spray, (2) material inoculated with mold and treated with bleach, and (3) material inoculated with mold and treated with Tilex solution. The data were matched on type of material and date of ELISA run and analyzed by using the Wilcoxon signed rank sum test (because of the nonnormality of the data). Statistical significance was defined as *P* value <.05. We tested the following hypothesis: H₀: difference = 0 (the treatment has no effect); H₁: difference $\neq 0$ (the treatment has an effect).

RESULTS

Loss of detection of *A fumigatus* antigen in solution by ELISA

Bleach treatment of *A fumigatus* conidia in an aqueous solution resulted in a significant decrease in *A fumigatus* antigen to levels below the limit of detection by ELISA compared with the untreated mold solution. Loss of ELISA activity was seen across a range of conidial concentrations from 0.63 to 10×10^6 conidia/mL. Fig 1 illustrates loss of ELISA activity with bleach treatment compared with mold alone at all mold concentrations tested (*P* = .03). Results were similar for Tilex treatment (data not shown).

Conidia counts and viability on building materials

To determine the viability of the conidia after treatment of the building material squares, we counted conidia numbers and cultured viable conidia. Culturability was used as the surrogate measure of viability. Table I provides results of conidia counts and culturability for 3 experiments. These same experiments yielded the solutions used in the ELISA below to determine antigenicity. Building material squares in the negative control groups, which were not inoculated with mold—groups 4 (no mold + water), 5 (no mold + bleach), and 6 (no mold + Tilex)—showed no detectable conidia by either micros-

| Condition | Experiment 1 | | Experimen | t 2 | Experiment 3 | | |
|---------------|--|----------------------|--|----------------------|--|------------------------|--|
| | Conidia count \times 10 ⁶ | $CFUs \times 10^{6}$ | Conidia count \times 10 ⁶ | $CFUs \times 10^{6}$ | Conidia count \times 10 ⁶ | CFUs × 10 ⁶ | |
| Plywood | | | | | | | |
| Mold + water | 6.1 | 0.6 | 17 | 0.7 | 0.08 | 0.4 | |
| Mold + bleach | 4.5 | 0.0055 | 25 | 0.001 | 0.19 | ND | |
| Mold + Tilex | 10.0 | ND | Not done | ND | 0.34 | ND | |
| Drywall | | | | | | | |
| Mold + water | 8.3 | 2.4 | 6 | 2.4 | 0.8 | 0.3 | |
| Mold + bleach | 6.8 | 0.01 | 2.2 | ND | 0.2 | ND | |
| Mold + Tilex | 9.7 | 2.3 | Not done | ND | 0.2 | ND | |
| OSB | | | | | | | |
| Mold + water | 7.5 | 1.7 | 33 | 0.1 | 10.0 | 3.0 | |
| Mold + bleach | 10.0 | 0.0001 | 6 | 0.0001 | 0.5 | ND | |
| Mold + Tilex | 10.0 | ND | Not done | ND | 9.5 | ND | |

TABLE I. Culturability of recovered A fumigatus conidia

ND, None detected.

copy or culture (data not shown). Aliquots from untreated building materials in group 1 (mold + water) showed from 0.1 to 33×10^6 conidia present. All untreated groups grew from 0.4 to $3.0 \times 10^6 A$ fumigatus colonies, although the numbers of culturable conidia were lower than those counted for all conditions. Building material squares treated with bleach or with Tilex had numbers of counted conidia similar to the positive controls (P = NS). However, bleach or Tilex treatment significantly reduced the number of cultured conidia (P = .003 and .011, respectively, compared with untreated mold conditions). This difference between counted and cultured A fumigatus suggests that although mold conidia were still present, they were nonviable. Mold antigen was also no longer detected on the treated boards by ELISA.

Loss of detection of *A fumigatus* on building materials by ELISA

Because we were concerned that dead *A fumigatus* conidia could still be allergenic, we investigated whether treated *A fumigatus* recovered from building materials would be detected by ELISA. Fig 2 shows the mean optical density results of ELISA tests conducted on 4 complete sets of building material squares. The results show that the *A fumigatus* binding capacity for the bleach-treated and Tilex-treated drywall and the bleach-treated and Tilex-treated OSB board are significantly lower compared with the untreated, mold-inoculated boards (P = .02 for both treatments). Mold did not grow well on plywood, and ELISA reactivity was unaffected by treatment with bleach or Tilex.

Morphologic changes with bleach and Tilex treatment

In addition to the loss of mold viability on bleach-treated and Tilex-treated building materials, the treated conidia appeared morphologically different from untreated conidia. Fig E1 (available in the Journal's Online Repository at www.mosby.com/jaci) compares bleach-treated with



FIG 2. Mean ELISA data for mold and inoculated building materials. *A fumigatus* antigen activity is expressed as mean OD from ELISA. Results compare bleach and Tilex treatment with untreated mold on 3 different building materials. Results are mean and SEM of n = 4.

untreated conidia using light microscopy. Scanning electron microscopy, as shown in Fig 3, demonstrates that the bleach-treated or Tilex-treated conidia are smaller and smoother, with loss of surface structures and some surface dimpling compared with healthy conidia.

Loss of skin test reactivity to bleach and Tilex-treated mold

A total of 21 individuals who reported that they were allergic to mold were enrolled in this study. Thirteen subjects did not react to either the Greer or the National Jewish extracts. Those tested also did not react to any of the treated building material extracts, indicating that they were nonirritating. Eight of the 21 were found to have a positive reaction to either the commercially available *A fumigatus* antigen or to antigen produced in our laboratory. The National Jewish *Aspergillus* antigen extract showed a higher concentration of Asp f 1 (1230 ng/mL) relative to the commercial extract (600 ng/mL). Four had a positive reaction to both, and 4 had a positive reaction to the National Jewish extract only (Table II). Of the



FIG 3. Scanning electron micrographs of untreated and treated *A fumigatus* conidia. Scanning electron micrograph of **(A)** untreated, **(B)** diluted bleach-treated, and **(C)** Tilex-treated *A fumigatus* conidia (10,000 \times) illustrates ultrastructural alterations of the conidial surface.

8 sensitized individuals, individual D was found to react to many of the skin prick test solutions in a way that suggested dermatographism. However, the skin prick test in this individual to the negative control was appropriately negative, and therefore, this case was included in our analysis as a valid case of Aspergillus allergy. We compared the skin test results of the mold extract treated with bleach or Tilex to the untreated National Jewish and commercial mold extracts. Bleach treatment inhibited the skin test response to A fumigatus in 5 of the 8 individuals, whereas Tilex inhibited the skin test response in 7 out of 8. Of the 4 individuals who reacted to the OSB and mold extract, 3 did not react to the bleach-treated mold extract, and 2 did not react to the Tilex-treated mold extract. Most subjects (7 of 8) did not react to either of the negative control extracts: OSB with water or OSB with Tilex. Six of 8 did not react to the OSB with bleach. Although these findings are consistent with the ELISA data for antigen inhibition above, the skin testing sample size is small, and this trend merits replication in a larger series.

Adverse reactions

None of the 21 study subjects had any major or minor adverse reactions to the skin testing procedure.

DISCUSSION

This study was designed to replicate real world exposures to fungal allergens and determine the efficacy of using standard, diluted household bleach or Tilex spray—under normal use conditions and at normal use concentrations—to control these exposures. The building materials, obtained from a lumberyard, are routinely used in building construction and are known to have the potential of becoming contaminated with mold. The *A fumigatus* used in this study is commonly associated with both building contamination and with allergy. Importantly, our results show that inhibition of mold growth and mold allergens is feasible under relevant environmental conditions and using commercially available building products.

Interestingly, we observed an order of magnitude difference between the numbers of *A fumigatus* conidia we counted by light microscopy and the number of CFUs we cultured. This suggests that 9 out of 10 *A fumigatus* are sterile. However, fungal allergens are intact in the untreated culture, as extracts elicited positive skin tests in subjects allergic to *A fumigatus*. When *A fumigatus* is treated with bleach or Tilex, the number of counted conidia remains the same. However, the mold is no longer viable, and importantly, surface allergens are no longer detected by ELISA or by skin prick testing. These findings suggest that treatment of *A fumigatus* mold by sodium hypochlorite not only kills mold but also effectively reduces the allergen load.

Differences observed in the sensitivity of the commercial versus National Jewish extracts are not surprising given the lack of standardization of mold extracts used in clinical skin testing.¹² Although we studied only a small number of patients, the data are consistent and supportive evidence of efficacy. These data warrant replication in a larger series of individuals and direct examination of health benefits.

One concern regarding the use of bleach solutions has been their loss of disinfectant capabilities in the presence of substantial amounts of organic material.^{1,5,13-17} A study by Bloomfield and Miller¹⁷ demonstrated substantial inactivation of sodium hypochlorite in the presence of blood. The presence of sputum slightly reduced the ability of sodium hypochlorite to kill *Mycobacterium tuberculosis*.¹⁴ In a subsequent study, Best et al¹⁶ found that high amounts of organic material and the presence of milk reduced the ability of sodium hypochlorite to kill *Listeria* monocytogenes. In general, the published literature suggests that sodium hypochlorite is a poor disinfectant on soiled surfaces and cautions that it is approved for use only on previously cleaned surfaces.⁵

Currently, some government agencies and mold contamination authorities recommend against the use of

| | Α | В | С | D | Е | F | G | н |
|--|---|---|---|---|---|---|---|---|
| Histamine | + | + | + | + | + | + | + | + |
| Saline | _ | _ | _ | _ | _ | _ | — | _ |
| Untreated A fumigatus commercial extract (Greer) | + | _ | _ | + | + | _ | _ | + |
| Untreated NJC A fumigatus extract | + | + | + | + | + | + | + | + |
| Bleach-treated NJC A fumigatus extract | + | _ | + | + | _ | _ | _ | _ |
| Tilex-treated NJC A fumigatus extract | _ | _ | _ | + | _ | _ | _ | _ |
| OSB, A fumigatus-inoculated, with water | _ | + | + | + | + | _ | _ | _ |
| OSB, A fumigatus-inoculated, with bleach | - | - | - | + | - | - | _ | _ |
| OSB, A fumigatus-inoculated, with Tilex | _ | _ | + | + | _ | _ | _ | _ |
| OSB, no mold, with water | _ | _ | _ | + | _ | _ | _ | _ |
| OSB, no mold, with bleach | _ | _ | + | + | _ | _ | _ | _ |
| OSB, no mold, with Tilex | _ | _ | _ | + | _ | _ | _ | _ |

NJC, National Jewish Center.

household bleach as a method of remediation in cases of fungal contamination of a building.⁵⁻⁷ The primary concerns regarding the use of chlorine bleach in this manner have included (1) the possible reduced capability of sodium hypochlorite to disinfect surfaces that have significant organic content and (2) the lack of published data testing whether bleach inhibits allergenic proteins on environmental surfaces. On the basis of the results of our study, sodium hypochlorite–containing products should be reconsidered as one of the tools in the remediation of mold-contaminated buildings.

This study provides an important proof of principle: sodium hypochlorite not only kills mold but also inhibits its antigenic properties. On the basis of these promising findings, additional research is warranted. Studies defining which surface molecules are denatured and removed from mold conidia by sodium hypochlorite may provide insights into the disinfectant's mechanism of action in inhibiting antigen. Field studies should be conducted to determine optimal application procedures, including application method, concentration, duration, and frequency of treatment in relation to duration of antigen inhibition and mold killing. Other types of surfaces that vary in their porosity and mold intrusion should be examined. Studies conducted in contaminated buildings may help us establish the real world methods, safety, and benefits of mold remediation performed either with or without sodium hypochlorite application. Although Aspergillus species are among the most common pathogenic molds found in contaminated buildings, it would be of interest to confirm that these results can be generalized to other common mold contaminants.

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