

Characterization of a monoclonal antibody (P40) against the 68 kD major allergen of *Penicillium notatum*

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Summary

A monoclonal antibody (MoAb P40) against the 68 kD major allergen of *Penicillium notatum* (*P. notatum*) was obtained by immunizing the mouse with a crude extract of *P. notatum*. Analysed by two-dimensional gel electrophoresis and immunoblotting, P40 reacted with two different isoforms of the 68 kD component of *P. notatum* with pIs of 5.4 and 5.5. In addition to *P. notatum*, P40 showed positive ELISA activity to *Aspergillus fumigatus* (*A. fumigatus*) but not to components of six other fungi including *Alternaria porri*, *Cladosporium cladosporioides*, *Aureobasidium pullulans*, *Fusarium solani*, *Rhizopus arrhizus* and *Candida albicans*. Analysed by ELISA, MoAb P40 also showed positive activity to two (*P. frequentans* and *P. roseopurpureum*) of the 10 other *Penicillium* species and two (*A. terreus* and *A. flavus*) of the four other *Aspergillus* species tested. SDS-PAGE and immunoblotting studies demonstrated P40 positive reactivity to components with MW of about 67 kD in all these *Penicillium* and *Aspergillus* species with positive ELISA activity to P40. Furthermore, immunoblotting activity of MoAb P40 to the 67 kD component of *A. niger* was also observed. The epitope of the 68 kD allergen of *P. notatum* recognized by MoAb P40 was resistant to treatment of periodate oxidation with concentration of NaIO₄ up to 20 mM. This MoAb may thus be useful in the characterization and purification of the 68 kD allergen from crude extracts, and in the molecular cloning of allergen genes.

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Introduction

Penicillium is a prevalent air-borne fungi and has been considered to be an important causative agent of extrinsic bronchial asthma [1–3]. Different species in the genus *Penicillium* may be associated with clinical allergy [1, 4–7]. However, little is known about the allergenic composition and the allergenic cross-reactivity of these *Penicillium* species. Monoclonal antibodies (MoAbs) against specific fungal antigens may provide a tool for studies on cross-reactivity among antigens of different fungi [8]. In the present study, we report the production of a monoclonal antibody (MoAb P40) against the 68 kD component of *Penicillium notatum* (*P. notatum*) which has been previously identified as a major allergen of this fungal species by sodium dodecyl sulphate-polyacrylamide gel electro-

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phoresis (SDS-PAGE) and immunoblotting using sera from 19 allergic patients and 20 blood donors [9]. The cross-reactivities among antigens of eight different genera of air-borne fungi and among 11 species of *Penicillium* and five different species of *Aspergillus* associated with clinical allergy were analysed using this MoAb. The epitope on the 68 kD component of *P. notatum* recognized by MoAb P40 has also been characterized in preliminary studies.

Materials and methods

Allergenic extracts

The ground and freeze-dried fungi preparations were obtained from Allergon AB (Engelholm, Sweden). They were 11 species of *Penicillium*, five species of *Aspergillus*, *Alternaria porri*, *Cladosporium cladosporioides*, *Aureobasidium pullulans*, *Fusarium solani*, *Rhizopus arrhizus* and

Candida albicans. The 11 *Penicillium* species used were *P. notatum*, *P. atramentosum*, *P. bifforme*, *P. brevicompactum*, *P. camembertii*, *P. digitatum*, *P. expansum*, *P. frequentans*, *P. godlewskii*, *P. roquefortii* and *P. roseopurpureum*. The five *Aspergillus* species used were *A. flavus*, *A. fumigatus*, *A. nidulans*, *A. niger* and *A. terreus*. Crude extracts of different fungi were prepared by extracting 5 g of each species with 100 ml of phosphate-buffered saline (PBS, containing 85 mM NaCl, 2.6 mM KH_2PO_4 , 49 mM Na_2HPO_4 , pH 8.0) for 36 hr at 4°C with stirring. After centrifugation, the supernatant solution was dialysed against phosphate buffer (10 mM, pH 7.2). After sterile filtration (0.2 µm; Sartorius, Hayward, California, U.S.A.), the extracts were lyophilized in aliquots and stored at 4°C. The protein content of crude extracts was determined by the dye-binding method of Bio-Rad (Richmond, California, U.S.A.).

Development of MoAbs

MoAbs against *P. notatum* were prepared essentially as described previously [10]. Spleen cells obtained from BALB/c mice immunized with the crude extract of *P. notatum* were fused with murine plasmacytoma NS-1 cells in the presence of polyethylene glycol (MW 1500 daltons; Merck, Hohenbrunn, Germany). Antibody-producing hybrid cells were screened by the enzyme-linked immunosorbent assay (ELISA) using the crude extract of *P. notatum*. Antigens of *P. notatum* recognized by MoAbs produced by hybridomata were analysed further by SDS-PAGE and immunoblotting. Hybridomata producing MoAbs against the 68 kD component of *P. notatum* were subcloned at least twice by limiting dilutions and then further expanded. The isotype of the MoAbs was determined by ELISA with class-specific antisera (Zymed Laboratories, San Francisco, California, U.S.A.).

ELISA

The ELISA was performed essentially as described previously [10]. The antigens used were crude extracts of different fungi which were coated separately onto wells of polyvinyl microtitre plates (Costar, Cambridge, Massachusetts, U.S.A.) by the addition of 50 µl of a 30 µg/ml solution of each fungal extract in carbonate buffer, pH 9.6. After blocking with 1% skim milk, culture supernatant solutions of hybridoma cells were added to each well and incubated for 1 hr at 37°C. After washing with PBS (150 mM, pH 7.4) containing 0.05% Tween 20 (PBST), alkaline phosphate-conjugated goat anti-mouse IgG (whole molecule; Sigma, St Louis, Missouri, U.S.A.) was added to each well and incubated for 1 hr at 37°C.

Wells were washed again with PBST and 50 µl of substrate solution (1 mg/ml *p*-nitrophenyl phosphate in 10% diethanolamine buffer, pH 9.8) was added to each well for 30 min at 37°C. Colour change was detected by measurement of the absorbance at 405 nm on a Titertek Multiskan (Flow Laboratories, McLean, Virginia, U.S.A.). Culture supernatant solutions of NS-1 cells and of hybridoma cells containing MoAbs against *Candida albicans* which have also been generated in our laboratory were used as negative control. Average of two readings from each of two separate determinations was calculated. Positive ELISA activity was defined as using hybridoma culture supernatant solutions which showed intensities of the absorbance greater than 1.5 times the absorbance obtained with the negative control supernatant solutions.

Gel electrophoresis and immunoblotting

The components of crude fungal extracts recognized by MoAbs produced or by IgE antibodies in sera of allergic patients were analysed by gel electrophoresis and immunoblotting essentially as described previously [9–13]. Briefly, components of the crude extracts were separated by SDS-PAGE or by the two-dimensional (2D) gel electrophoresis and transferred electrophoretically onto polyvinylidene difluoride membrane (0.45 µm, Millipore, Bedford, Massachusetts, U.S.A.). After blocking with 1% skim milk, the blot was incubated with culture supernatant solutions of hybridoma cells or with human serum samples and monoclonal anti-human IgE antibodies developed in our laboratory [14]. After washing procedures, all the blots were incubated with affinity-purified, horseradish peroxidase-labelled goat anti-mouse IgG (H+L) (BioRad) for 1 hr at room temperature. After washing, the blot was developed in a substrate solution of acetate buffer (50 mM, pH 5.0) containing 1 mM 3-amino-9-ethyl-carbazole (Sigma) and 0.015% (v/v) hydrogen peroxide for about 5 min. After washing with water, the deposition of the red stain which demonstrated antigen-antibody binding was recorded by photography. Culture supernatant solutions of NS-1 cells and of hybridoma cells containing MoAbs against *Candida albicans* generated in our laboratory and cord serum samples were used as negative control.

Periodate oxidation

Periodate oxidation of antigens was performed as described by Woodward *et al.* [15]. The antigens of *P. notatum* coated onto microtitre plates were exposed to varying concentrations of sodium *m*-periodate (0, 0.1, 0.5, 1.0, 5.0, 10 and 2 mM; May & Baker, Dagenham, England). The activity of MoAbs to antigens treated by periodate oxidation was then determined by ELISA.

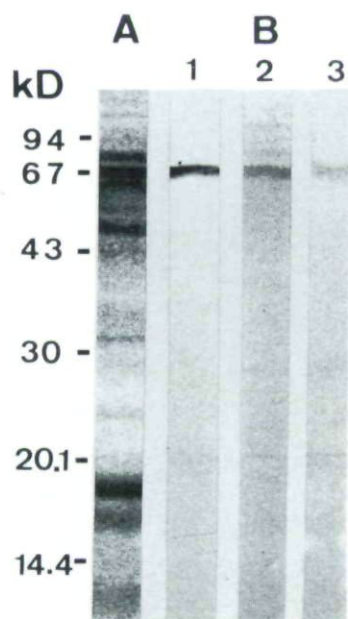


Fig. 1. Immunoblot analysis of antibody activities to components of *P. notatum*. (A) Coomassie blue-stained protein profile of the crude extract of *P. notatum* separated by SDS-polyacrylamide gel electrophoresis. (B) Lane 1 shows the immunoblotting activity of MoAb P40 to the crude fungal extract. Lanes 2 and 3 show the immunoblotting activity of IgE antibodies in sera of two allergic patients to the same fungal extract.

Results

Hybridomata were obtained by fusion of immunized murine spleen cells with plasmacytoma NS-1 cells. Hybridomata secreting MoAbs against *P. notatum* were first screened by ELISA. In order to identify antigens recognized by antibodies produced, protein components in the crude extract of *P. notatum* were separated by SDS-polyacrylamide gel electrophoresis. Figure 1(A) shows that there were at least 30 different components with molecular weights (MWs) ranging from more than 100 to about 10 kD in the crude extract of *P. notatum*. One hybridoma (P40) producing MoAbs which showed immunoblotting activity to the 68 kD component of *P. notatum* (Fig. 1, B-1) was finally chosen and expanded. The isotype of MoAb P40 is IgG1, k as analysed by ELISA using class-specific antisera. The same 68 kD component of *P. notatum* also showed immunoblotting activity to IgE antibodies in sera from two allergic patients with positive immediate skin test to *P. notatum* (Fig. 1, B-2 and B-3). In order to characterize further antigens recognized by MoAb P40, 2D gel electrophoresis and immunoblotting was performed. Figure 2(a) shows the silver-stained two-dimensional protein profile of the crude extract of *P. notatum*. MoAb P40 showed immuno-

blotting activity to two different isoforms of the 68 kD component of *P. notatum* with pI values of 5.4 and 5.5 (Fig. 2b).

For analysis of cross-reactivity among antigens of different fungi, MoAb P40 showed positive ELISA activity to *A. fumigatus* but not to components of six other fungal genera including *Alternaria porri*, *Cladosporium cladosporoides*, *Aureobasidium pullulans*, *Fusarium solani*, *Rhizopus arrhizus* and *Candida albicans*. Furthermore, among the 11 different species of *Penicillium* and five different species of *Aspergillus* tested, MoAb P40 showed positive ELISA activity only to antigens in the crude extracts of *P. notatum*, *P. frequentans*, *P. roseopurpureum*, *A. fumigatus*, *A. terreus*, and *A. flavus* (Table 1). The cross-reactivity among different *Penicillium* and *Aspergillus* species was also analysed by SDS-PAGE and immunoblotting. Figures 3 and 4 show the Coomassie blue-stained SDS-PAGE protein profiles of 11 species of *Penicillium* and five species of *Aspergillus*, respectively. Among all the *Penicillium* and *Aspergillus* species tested, MoAb P40 showed immunoblotting activity to components with MW of about 67 kD in the crude extracts of *P. notatum*, *P. frequentans*, *P. roseopurpureum*, *A. fumigatus*, *A. terreus*, *A. flavus* and *A. niger* (Fig. 5 and Table 1). In addition, relatively weak reactivity of MoAb P40 to components of *P. frequentans* and *P. roseopurpureum* with MW of about 64 kD has also been detected (Fig. 5A, lanes 2 and 3). For negative control, no colour development was observed when culture supernatant solutions from NS-1 cells or from hybridomata producing MoAbs against *Candida albicans* and cord serum samples were used in the immunoblot analysis.

The epitope on the 68 kD component of *P. notatum* recognized by MoAb P40 was resistant to treatment of periodate oxidation with concentration of NaIO_4 up to 20 mM (data not shown).

Discussion

In clinical practice, mould-sensitive patients often demonstrate immediate skin reactivity to a variety of fungi [16–18]. It is not clear, however, whether such multiple reactions occur by mere coincidence, or if moulds share common antigenic/allergenic determinants [19]. Cross-reactivity between antigens of *Penicillium* and those of *Alternaria*, *Aspergillus*, *Fusarium* and *Cladosporium* has been demonstrated [20–21]. In the present study, a MoAb (P40) against the 68 kD major allergen of *P. notatum* was generated and used on studies of cross-reactivity among different fungal species. Among eight different genera of fungi tested, antigenic cross-reactivity was detected only between antigens of closely related *Penicillium* and *Aspergillus* species which belong to the

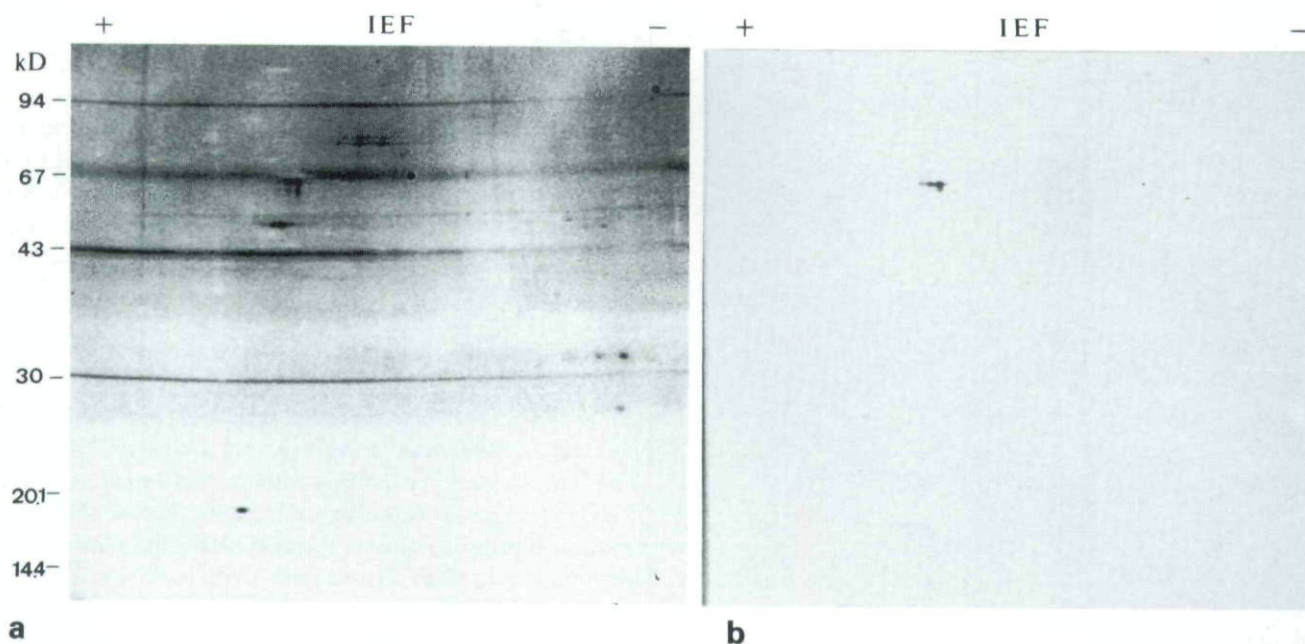


Fig. 2. Immunoblot analysis of antibody activity of MoAb P40 to components in the crude extract of *P. notatum* separated by two-dimensional gel electrophoresis. (a) Silver-stained two-dimensional protein profile of the crude extract of *P. notatum*. (b) Immunoblotting activity of MoAb P40 to components in the crude extract of *P. notatum* separated by two-dimensional gel electrophoresis.

Table 1. Reactivities of MoAb P40 to different species of *Penicillium* and *Aspergillus* analysed by ELISA and SDS-PAGE-immunoblotting

Mould species	ELISA	SDS-PAGE-immunoblotting
<i>Penicillium</i> species		
<i>P. notatum</i>	+	+
<i>P. frequentans</i>	+	+
<i>P. roseopurpureum</i>	+	+
<i>P. roquefortii</i>	—	—
<i>P. biforme</i>	—	—
<i>P. camembertii</i>	—	—
<i>P. atramentosum</i>	—	—
<i>P. digitatum</i>	—	—
<i>P. expansum</i>	—	—
<i>P. brevicompactum</i>	—	—
<i>P. godlewskii</i>	—	—
<i>Aspergillus</i> species		
<i>A. fumigatus</i>	+	+
<i>A. terreus</i>	+	+
<i>A. flavus</i>	+	+
<i>A. niger</i>	—	+
<i>A. nidulans</i>	—	—

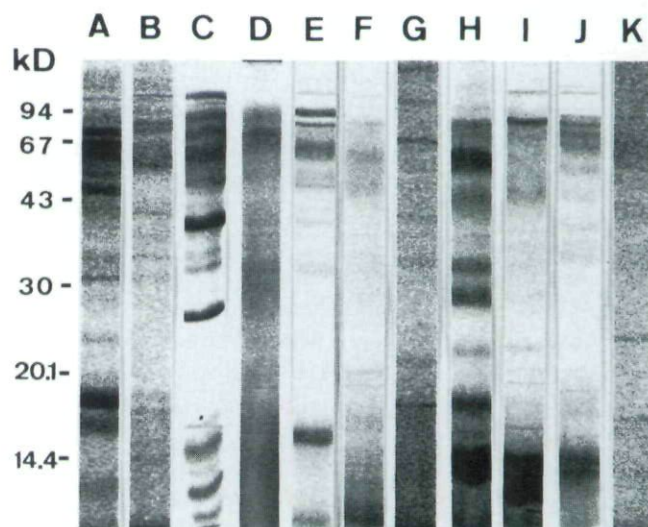


Fig. 3. The Coomassie blue-stained SDS-PAGE protein profiles of the crude extracts of 11 *Penicillium* species. The 11 *Penicillium* species analysed were *P. notatum* (A), *P. frequentans* (B), *P. roseopurpureum* (C), *P. roquefortii* (D), *P. biforme* (E), *P. camembertii* (F), *P. atramentosum* (G), *P. digitatum* (H), *P. expansum* (I), *P. brevicompactum* (J) and *P. godlewskii* (K).

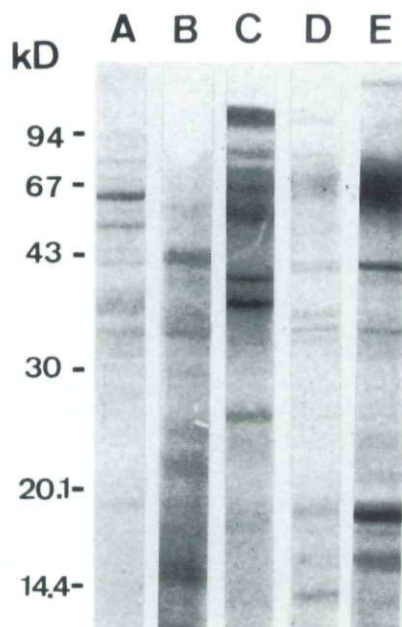


Fig. 4. The Coomassie blue-stained SDS-PAGE protein profiles of the crude extracts of five *Aspergillus* species. The five *Aspergillus* species analysed were *A. fumigatus* (A), *A. terreus* (B), *A. niger* (C), *A. flavus* (D) and *A. nidulans* (E).

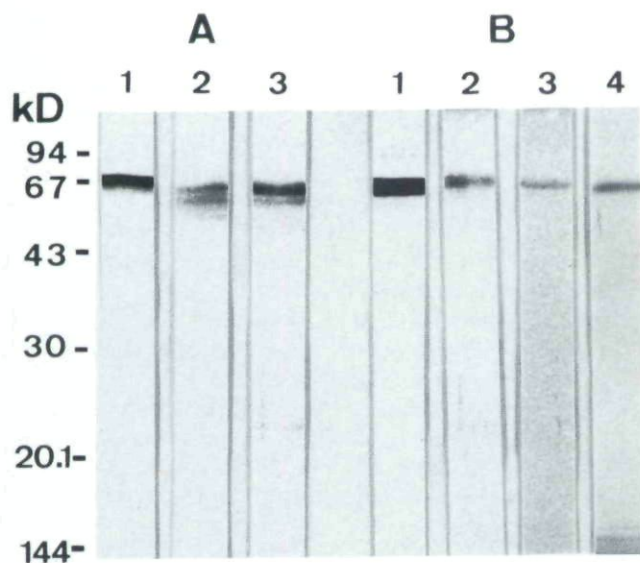


Fig. 5. Immunoblot analysis of antibody activity of MoAb P40 to components in three species of *Penicillium* and four species of *Aspergillus*. Lanes 1–3 in (A) represent results of immunoblotting using crude extracts of *P. notatum* (1), *P. frequentans* (2) and *P. roseopurpureum* (3). Lanes 1–4 in (B) show the results when crude extracts of *A. fumigatus* (1), *A. terreus* (2), *A. niger* (3) and *A. flavus* (4), respectively, were used.

same family of *Moniliaceae* [22–23]. This finding correlates with the claim that although common antigenic/allergenic determinants have been shown to occur within and across generic lines, the degree of cross-reactivity is low between members of different fungal genera [19].

Among different species of *Penicillium*, MoAb P40 showed positive ELISA and immunoblotting activities only to three (*P. notatum*, *P. frequentans* and *P. roseopurpureum*) of the 11 *Penicillium* species tested. For analysis of cross-reactivity, Nemergut *et al.* reported that by immunodiffusion using rabbit antisera, there was cross-reactivity between *P. notatum* and *P. chrysogenum*, but no cross-reactivity between other species of *Penicillium* (*P. citrinum*, *P. italicum*, *P. roqueforti*) was detected [24]. Data from these results suggests that different allergenic compositions may exist in different *Penicillium* species. Thus, it is important to analyse allergenic components of important *Penicillium* species associated with clinical allergy and to select the proper species for clinical use.

Results from this study also showed that MoAb P40 exhibited positive ELISA and immunoblotting activities to antigens of *A. fumigatus*, *A. terreus* and *A. flavus*. Analysed by SDS-PAGE and immunoblotting, the 67 kD component of *A. fumigatus* recognized by MoAb P40 also showed positive reactivity to IgE antibodies in serum of one allergic patient with positive immediate skin test to *Aspergillus* species (data not shown). This suggests that both *P. notatum* and *A. fumigatus* share at least one allergenic component with similar MW and antigenic epitope. Concerning the cross-reactivity within the genus *Aspergillus*, it has been reported that there appear to be common antigens within the *fumigatus* and *niger* groups, which are antigenically distinct from the *versicolour*, *nidulans* and *glaucus* groups [25]. In the present study, in addition to reactivity to *A. fumigatus*, *A. terreus* and *A. flavus*, MoAb P40 also showed positive immunoblotting activity to the 67 kD component of *A. niger*. No activity of MoAb P40 to antigens of *A. nidulans* was detected analysed by both ELISA and immunoblotting. The discrepant results obtained concerning cross-reactivity between *P. notatum* and *A. niger* analysed by ELISA and SDS-PAGE-immunoblotting may be due to some limitations of techniques used such as the presence of fungal components which fail to adsorb onto microtitre plate wells. Furthermore, pre-treatment of sample proteins with sample buffer containing SDS and 2-mercaptoethanol before SDS-PAGE may contribute to another reason for the discrepancy observed.

Mild periodate oxidation at acid pH has been shown to cleave carbohydrate vicinal hydroxyl groups without altering the structure of polypeptide chains [15]. In this study, the epitope of the 68 kD allergen of *P. notatum* recognized by MoAb P40 was resistant to the treatment of

periodate oxidation, it may thus not be associated with carbohydrates and can be used in the molecular cloning of the gene of the 68 kD major allergen of *P. notatum*.

In conclusion, by using MoAb P40 against the 68 kD major allergen of *P. notatum*, antigenic/allergenic cross-reactivities between three different species of *Penicillium* (*P. notatum*, *P. frequentans*, *P. roseopurpureum*) and four different species of *Aspergillus* (*A. fumigatus*, *A. terreus*, *A. flavus* and *A. niger*) have been demonstrated. The allergenic cross-reactivity described should be considered in the diagnostic clinical allergy.

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