

Modified *par j* I allergen from *P. judaica* pollen and its rate of absorption in rats

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1. Summary

Polymerized allergens (allergoids) have been introduced in the immunotherapy of allergic disease in order to reduce the risk of side effects. However, their high molecular weight can be a limit, particularly when they are administered by a route involving passage through the mucosal barrier. We describe a simple procedure aimed at developing an original modified allergen with significantly less allergenic potential (intended as human IgE-binding capacity) but preserving the monomeric nature of the molecule. *Par j* I, the major allergen of *Parietaria judaica* pollen, was purified by a combination of monoclonal antibodies and affinity chromatography. *Par j* I allergen was then modified by reaction with potassium cyanate (KCNO), and compared with the native allergen to evaluate its allergenic potency (RAST-inhibition) and molecular weight (SDS-PAGE). Modified allergen showed significantly lower allergenic potency but kept its original molecular weight, making it particularly suitable for buccal (sublingual) administration. To study the adsorption profile, modified *Par j* I was radiolabeled and administered intravenously and sublingually to normal rats. The prospects for clinical application of the modified allergen are discussed.

2. Introduction

IgE antibodies play an important role in mediating allergic reactions. The beneficial effects of immu-

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notherapy in allergic diseases have been well documented [1]. An important advantage of immunotherapy compared to pharmacological treatment, known to limit its effect only on symptoms, may lie in its interference with the pathophysiological mechanisms causing the disease [2,3]. It is undeniable, however, that immunotherapy sometimes involves the risk of severe side effects [4]. Chemically modified allergens (allergoids) with reduced allergenic capacity have been developed in order to obtain safe and effective products [5] and the allergen modified with glutaraldehyde has been extensively studied. In this case, the chemical modification considerably increases the molecular weight [6,7].

Another way of improving the safety of immunotherapy and making it more convenient to the patient is to use routes of administration other than the subcutaneous (s.c.) one. Sublingual administration of the allergenic extract seems a good alternative [8-10], enabling patients to comply better because they can self-administer the treatment at home. The mechanism underlying the clinical benefits of this route is not yet understood although it probably involves the passage of allergenic components through the mucosal tissue in the mouth. In this case polymerized allergens ought to be hindered in their transmucosal passage, in view of their molecular weight, but native allergens on the other hand could be associated with a risk of side effects as they reach the systemic circulation rapidly.

The present report describes the purification of *Par j* I, the major allergen of *Parietaria judaica* pollen extract [11], its chemical modification by an original procedure, which does not change its molecular weight reducing its allergenic potential, and its rate of adsorption as ¹²⁵I-labeled protein when administered by the sublingual route to normal rats.

We chose *Parietaria* pollen because it is a major cause of allergic respiratory disease in the Mediterranean area [12].

3. Materials and Methods

3.1. Preparation of *Parietaria judaica* (Pj) pollen extract

Pj pollen (50 g) was submitted to 5% aqueous extraction in 5 mM ammonium bicarbonate overnight at 4°C under stirring. The suspension was centrifuged at $20\,000 \times g$ for 30 min at 4°C; the supernatant was filtered through a 0.22 μ m filter then gel-filtered on a Sephadex G25 column (Pharmacia, Milan, Italy) equilibrated with 0.02 M, PBS, pH 7.2. The excluded peak was collected and characterized for its protein content and allergenic potency by the BioRad method (BioRad, Milan, Italy) and RAST-inhibition technique, respectively.

3.2. Production of monoclonal antibodies

Balb/c mice were immunized i.p. with 100 μ g, as protein content, of Pj extract, followed by 4 booster doses at intervals of 3 weeks. Three days after the last immunization, spleen cells were used for fusion according to Koehler and Milstein [13]. Growth of hybrid clones was observed in 125 of 450 wells, and supernatants from 18 of these reacted in ELISA against the Pj extract. Clones with an optical density greater than 2.0 were expanded in vitro and supernatants of some of them were further characterized in immunoblotting experiments.

3.3. ELISA

Wells of microtiter plates were coated overnight with 5 μ g of Pj extract diluted in 100 μ l of 0.1 M carbonate buffer, pH 9.6. After saturation with 100 μ l of 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), the sensitized plates were incubated 1 h with 100 μ l of culture supernatant and monoclonal antibodies (mAb) bound were detected by incubating for 1 h with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin serum (Sigma, Milan, Italy). Peroxidase activity was detected using hydrogen peroxide/*o*-phenylene-diamine as substrate and absorbance at 492 nm was read after 30 min using a microplate spectrophotometer (Multiscan, Flow, Milan, Italy).

3.4. SDS-PAGE and immunoblotting

Electrophoresis was carried out according to Laemmli [14] in a linear 15% polyacrylamide gel using an SE-250 apparatus (Hoefer, Prodotti Gian-

ni, Milan, Italy). Samples were diluted in Tris buffer containing 5% β -mercaptoethanol and 2% SDS and boiled for 5 min. In a first run molecular weight markers and Pj extract were loaded and, after 3 h at 30 A, resolved components were blotted by a semi-dry electroblotter on nitrocellulose paper. Transferred proteins were incubated with supernatants of ELISA-positive clones or with unrelated ascites (anti-mites extract) and then with a peroxidase-conjugated goat anti-mouse IgG serum. The signal was developed using enhanced-chemiluminescence reagents (ECL Western Blotting, Amersham, Amity, Milan, Italy) according to the manufacturer's instructions.

The clone designated P2C8, producing mAbs against a Pj component known to have a molecular weight of 12.5 kDa [15], was expanded in vivo in Balb/c mice, and IgG fraction from ascitic liquid, obtained by salt precipitation (50% sulphate ammonium saturated solution), was used as ligand in immunoaffinity. No band was visible when a supernatant from an unrelated clone was tested.

A second electrophoresis was performed loading molecular weight markers, Pj extract, affinity-purified fraction and modified *Par j* I. The run was carried out as before, and resolved protein was stained with Coomassie Brilliant Blue R250.

3.5. Purification of *Par j* I by affinity chromatography

In order to prepare immunoabsorbent P2C8, the IgG fraction purified by salt precipitation was coupled with CNBr-activated Sepharose (Pharmacia, Milan, Italy) according to the manufacturer's instructions. Pj extract was applied over the immunoabsorbent column equilibrated with PBS, and bound fraction was eluted with 5 mM glycine in 50% ethylene glycol, pH 10, dialyzed against 20 mM phosphate buffer pH 6.8 and concentrated on a cellulose ultrafiltration membrane with a nominal cut-off of 3.5 kDa. Purity and allergenic potency of the eluate were established by SDS-PAGE and RAST inhibition, respectively.

3.6. Preparation of chemically modified *Par j* I (mPar j I)

The affinity-purified fraction from Pj extract was diluted at 1 mg/ml in 20 mM phosphate buffer, pH 6.8. Then recrystallized KCNO and sodium tetraborate at final concentrations of 0.5 M and 0.1 M, respectively, were added, to a pH of 9.3. The solution was left for 20 h in a water bath at 40°C and afterwards gel-filtered on a Sephadex G25 column (Phar-

macia, Milan, Italy) equilibrated with 0.02 M PBS to remove excess salt. Because of the effect of gel filtration, the excluded peak (mPar j I) collected is double the initial one, bringing the concentration of the sample to 0.5 mg/ml.

The allergenic potency of mPar j I was tested by RAST-inhibition, in comparison with the Pj extract and non-modified allergen. mPar j I was radiolabeled with ^{125}I according to the Iodogen method (Pierce, Milan, Italy). Labeled proteins were separated from free ^{125}I by gel filtration chromatography on a Sephadex G25 column.

3.7. RAST-inhibition

RAST-inhibition analysis was performed according to Yman et al. [16], with some modifications. Briefly, 30 μl of serial dilutions of Pj extract or affinity-purified Par j I or mPar j I (initial concentration 0.5 mg/ml for all samples) were mixed in tubes with 20 μl of a pool of sera (with specific IgE: class 3 and 4 as RAST value) from patients allergic to *P. judaica* pollen. Positive and negative controls consisted of 30 μl of PBS pre-incubated with either the same pool or a normal serum (without specific IgE) respectively. After 2-h incubation, a polystyrene bead sensitized with Pj extract (Sferikit, Lab. Farmaceutico Lofarma, Milan, Italy) was added to each tube and incubated overnight.

Bound IgE was detected using 50 μl of an ^{125}I -labeled anti-IgE antibody. The residual radioactivity was measured in a gamma scintillation counter. Data were expressed as the percentage of inhibition as follows:

$$\% \text{ inhibition} = \frac{(\text{cpm positive control}) - (\text{cpm sample})}{(\text{cpm positive control}) - (\text{cpm negative control})} \times 100$$

and were plotted and evaluated according to the FDA protocol [17]. The sample volume (μl) able to inhibit the RAST response by 50% was defined as C_{50} and this parameter, inversely correlated with allergenic potency, was used to compare the different allergens.

3.8. Experimental design

Twenty male Sprague-Dawley rats (Charles River, Calco, Como, Italy) weighing 300–325 g were treated with atropine (1 mg/kg subcutaneously) to reduce salivation, and were anesthetized and incannulated with polythene catheters in the right atrium through

the jugular vein. After a basal blood sample, rats were divided into two groups: the first received 1.5 μCi of radiolabeled mPar j I (specific activity 46 $\mu\text{Ci}/\mu\text{g}$) in 500 μl of saline i.v., and the second was treated by the sublingual route (15.0 μCi in 50 μl of the same solution).

Blood from each animal was sampled at 15 and 30 min, 1, 2, 4, 8 and 24 h in tubes containing 0.15 M EDTA, then centrifuged at 3000 rpm at 4°C. Portions of 150 μl of plasma from each sample were counted in a gamma-counter.

4. Results

4.1. Allergenic activity (human IgE binding) of modified and native forms of Par j I

The allergenic potency of native and mPar j I was compared by RAST-inhibition experiments. The C_{50} for mPar j I (3.968 μl) was significantly higher than for the native form (0.009 μl) (Fig. 1), indicating that the chemical modification of Par j I by reaction with KCNO considerably reduces (1/440) its allergenic activity.

From Fig. 1 we can therefore deduce that Par j I has high clinical relevance because by itself it inhibits the response of human IgE antibodies to the various different allergens present in Pj extract ($C_{50} = 0.003 \mu\text{l}$).

4.2. Characterization of the mPar j I molecule

Chemical modification by KCNO is based on the transformation into a ureido group [18] of ϵ -amino groups of the lysine moiety present in the Par j I molecule. The degree of the substitution was checked according to Fields [19] and was more than 85% (data not shown).

In order to verify that our chemical procedure did not change the molecular weight of Par j I, we compared the native and the modified allergen by SDS-PAGE analysis. Native Par j I showed a band with the same molecular weight as mPar j I, confirming that reaction with KCNO, while reducing allergenic potency, did not influence the size of derived molecule (Fig. 2).

4.3. Recovery of ^{125}I -labeled mPar j I in plasma of rats after intravenous and sublingual administration

The kinetic profiles were compared for the two routes. Data are representative of two different experiments. After sublingual administration, radioac-

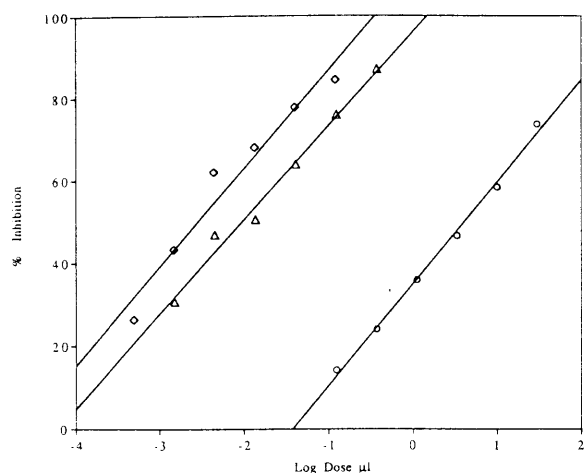


Fig. 1. Linear fittings of RAST-inhibition data for the Pj extract (□), affinity purified *Par j* I (△) and *mPar j* I (○). All samples were tested starting from a protein concentration of 0.5 mg/ml. Data are the mean of 3 replicates.

tivity was recovered in plasma of normal rats at time-related increasing levels, reaching a maximum 8 h after treatment (Fig. 3A). In contrast, after i.v. ^{125} I-labeled *mPar j* I, radioactivity quickly declined in the systemic circulation, disappearing almost completely in about 2 h (Fig. 3B).

4. Discussion

Immunotherapy by s.c. injection of allergenic extracts has been used for decades in patients with allergic symptoms [1]. However, the risk of side effects has led the researchers to explore safer approaches. Polymerized allergens with glutaraldehyde or formal-

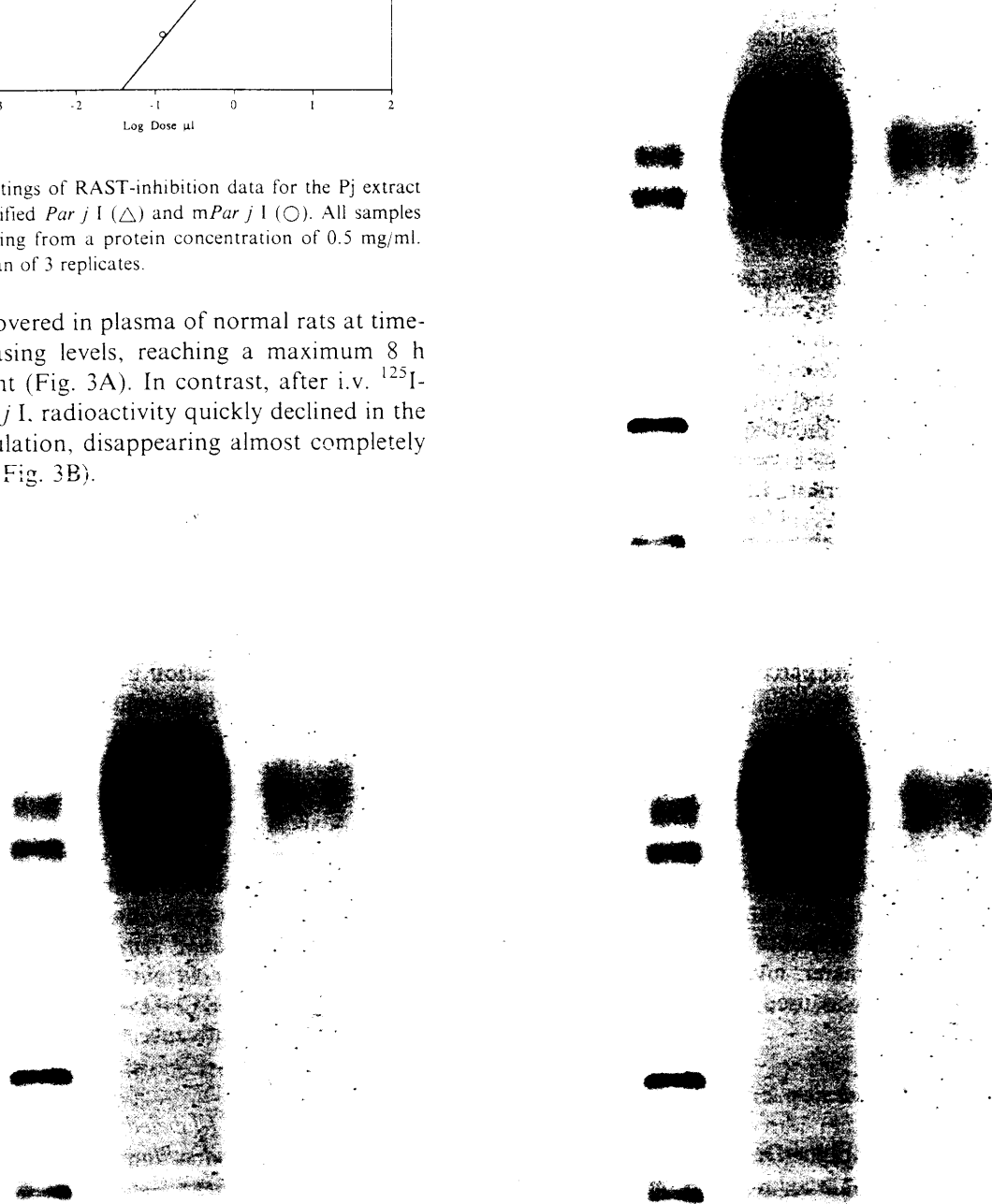


Fig. 2. SDS-PAGE analysis of purified *Par j* I (1 mg/ml, lane A), Pj extract (1 mg/ml, lane B) and *mPar j* I (0.5 mg/ml, lane D). Molecular weight markers: phosphorylase b, 97 kDa; BSA, 67 kDa; myoglobin, 17 kDa; and lysozyme, 14 kDa (lane C).

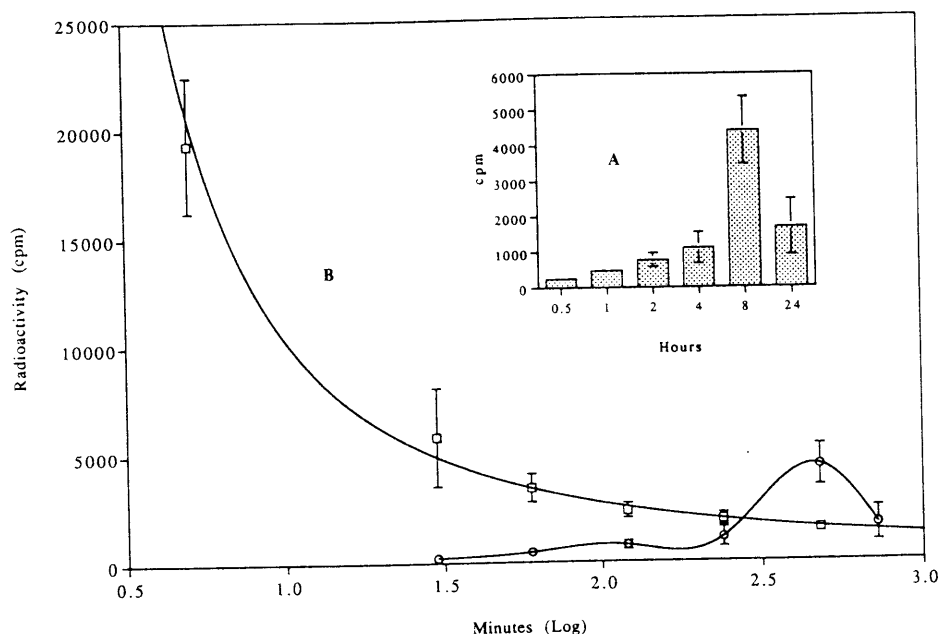


Fig. 3. Kinetic profiles (B) based on recovery of radioactivity after sublingual (○) or intravenous (□) administration of ^{125} I-labeled mPar j I allergen. Panel A simply shows the details, as histograms, of the absorption pattern after buccal treatment of rats with radiolabeled mPar j I. Data are the mean of 2 experiments each using 10 rats.

dehyde referred to as *allergoids*, are recognized as an effective form of treatment, with a lower incidence of local and immediate type reactions [5]. Allergoids are characterized by a marked reduction of allergenic potency related with an increase of the relative molecular weight (10^5 – 10^6 Da) [6,7].

Recently, *hyposensitization* to allergens has been achieved administering allergenic extracts by routes other than the s.c. one. Sublingual administration appears to offer an acceptable therapeutic option [8–10], although further studies are required to check the real incidence of side effects. Considering that the molecular size of a substance can hinder its ability to cross biological membranes, it is probable that polymerized allergens would encounter obstacles in passing through mucosal tissues, so they do not lend themselves to local application via nasal, bronchial or buccal routes. Sublingual administration, however, is a safe and effective method of giving drugs; the drug is absorbed, not subject to first-pass metabolism, and released over several hours [20].

Evidence of the presence of the allergen in the systemic circulation after administration of the allergenic extract has been published by Leeling et al. [21] who treated rats with radiolabeled allergen by s.c. injection. Nothing is known about the pattern of absorption after sublingual treatment. One reason which makes it difficult, if not impossible, to study the kinetic pattern is the considerable heterogeneity

of allergenic extracts. In the case of *P. judaica* pollen, the extract is a mixture of several different components with a molecular weight ranging from 10 to 90 kDa, as demonstrated by SDS-PAGE. In view of this we assumed that it would be useful to start from a purified allergen to gain a better understanding of the kinetic pattern. We therefore developed the mAbs against the major allergen, named *Par j* I [22], and used affinity chromatography to purify *Par j* I and characterize its molecular weight (about 12.5 kDa).

The clinical relevance of *Par j* I is clear from the RAST-inhibition data. By itself *Par j* I ($C_{50} = 0.009$) induced about 33% of the response, in terms of inhibition, obtainable with *Pj* in toto ($C_{50} = 0.003$).

The chemical effect of KCNO on proteins generally involves selective carbamylation of ϵ -aminogroups of lysine residues transforming them into ureido groups [18]. A similar procedure was used to modify a mixture of allergenic proteins [23]. An important finding of the present work is that this simple chemical modification, when applied to *Par j* I allergen, gives rise to a very particular protein derivative with significantly lower allergenic activity while preserving its monomeric nature. These features distinguish mPar j I allergoid from others used, whose molecular size considerably increases as a consequence of the polymerization induced by chemical reaction with aldehydes.

Our findings and the recovery of radioactivity in-

icated after buccal administration mPar j I is absorbed to a kinetic pattern with slow releases of the allergen over some hours (peak after about 8 h). Although we cannot exclude it, the possibility of part of the radioactivity being due to ingestion of radiolabeled allergen is unlikely as the rats were anesthetized and treated with atropine to reduce salivation, both measures being intended to limit swallowing.

Comparing the kinetic profile after i.v. and sublingual mPar j I and correcting for the difference in the amounts of radiolabeled allergen injected, the bioavailability of the allergen, after sublingual treatment, determined by the weighing method of the area under the blood-levels time curve [24], was approximately 1% of that after i.v. injection. Despite the small amount of allergen detected, our observation of its absorption in vivo introduces a new element of discussion about the possible mechanism by which immunotherapy based on sublingual administration of allergenic extracts acts. According to Scadding and Brostoff [9] the mechanism of action may reflect the desensitization of basophils; Holt et al. [25] sustain that, at least in normal rats, the stimulation of allergen-specific suppressor cells in the regional lymph nodes draining the oral cavity plays an important role.

The clinical relevance of our finding remains to be established. Our kinetic studies were done on non-sensitized animals. This differs from the clinical situation, particularly at the level of the oral cavity, where the saliva of atopic patients contains specific IgE antibodies [26]. The presence of these antibodies could, on the one hand, limit transmucosal passage of an allergen administered by the sublingual route and, on the other, amplify a potential local effect. Further studies of any humoral immune responses (IgG, IgA) induced by this modified allergen when administered by the sublingual route are obviously essential before drawing any firm conclusions, but our data suggest it might be a serious candidate for immunotherapy of allergic diseases.

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The image is a dark, grainy, high-contrast scan, likely of a document page. It features a large, solid black rectangular area in the center, which appears to be a redacted section or a very dark image. To the right of this black area, there is a lighter, textured region that looks like a scan of a white surface with some noise or a different material. The overall image has a very high level of contrast and a noisy, grainy texture, characteristic of a poor quality scan or a heavily processed image.

A	B	C	D
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Fig. 2. SDS-PAGE analysis of purified *Par j* I (1 mg/ml, lane A), Pj extract (1 mg/ml, lane B) and m *Par j* I (0.5 µg/ml, lane D). Molecular weight markers: phosphorylase b, 97 kDa; BSA, 67 kDa; myoglobin, 17 kDa; and lysozyme, 14 kDa (lane C). Lane A: Sporozoite; Lane B: Sporozoite; Lane C: Sporozoite; Lane D: Sporozoite antigen, Cytokine, Vaccine (Graftman).

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