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Effect of chemical modifications on allergenic potency of peanut proteins

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ABSTRACT

Background: *Modification of native peanut extracts could reduce adverse effects of peanut immunotherapy.*

Objective: *We sought to compare native and chemically modified crude peanut extract (CPE) and major peanut allergens Ara h 2 and Ara h 6 in a mediator-release assay based on the rat basophilic leukemia (RBL) cell line transfected with human Fcε receptor.*

Methods: *Native Ara h 2/6 was reduced and alkylated (RA), with or without additional glutaraldehyde treatment (RAGA). CPE was reduced and alkylated. Sera of subjects with peanut allergy (16 males; median age 7 years) were used for overnight RBL-passive sensitization. Cells were stimulated with 0.1 pg/mL to 10 μg/mL of peanut. β-N-acetylhexosaminidase release (NHR) was used as a marker of RBL degranulation, expressed as a percentage of total degranulation caused by Triton X.*

Results: *Median peanut-specific immunoglobulin E was 233 kU_A/L. Nineteen subjects were responders, NHR ≥ 10% in the mediator release assay. Responders had reduced NHR by RA and RAGA compared with the native Ara h 2/6. Modification resulted in a later onset of activation by 10- to 100-fold in concentration and a lowering of the maximum release. Modified RA-Ara h 2/6 and RAGA-Ara h 2/6 caused significantly lower maximum mediator release than native Ara h 2/6, at protein concentrations 0.1, 1, and 10 ng/mL ($p < 0.001$, < 0.001, and < 0.001, respectively, for RA; and < 0.001, 0.026, and 0.041, respectively, for RAGA). RA-CPE caused significantly lower maximum NHR than native CPE, at protein concentration 1 ng/mL ($p < 0.001$) and 10 ng/mL ($p < 0.002$). Responders had high rAra h 2 immunoglobulin E (mean, 61.1 kU_A/L; $p < 0.001$) and higher NHR in mediator release assay to native Ara h 2/6 than CPE, which indicates that Ara h 2/6 were the most relevant peanut allergens in these responders.*

Conclusions: *Chemical modification of purified native Ara h 2 and Ara h 6 reduced mediator release in an in vitro assay ~100-fold, which indicates decreased allergenicity for further development of the alternative candidate for safe peanut immunotherapy.*

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Peanut allergy affects >1% of young children in the developed countries.[1] Peanut is the major cause of severe and fatal food-induced anaphylaxis.[2,3] Currently, there is no cure for peanut allergy.[4] Prior studies demonstrated efficacy of subcutaneous peanut immunotherapy with crude peanut extract (CPE), however, with an unacceptable rate of serious adverse reactions.[5] Therefore, novel approaches for peanut immunotherapy are desirable.[6–9] Chemical modification could represent an effective strategy for adverse effect reduction in peanut immunotherapy.

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In the United States, immunoglobulin (Ig) E antibodies to Ara h 1 and to Ara h 2 and its homolog, Ara h 6, were most often detected in subjects who were 90–100% peanut reactive, and were associated with increased risk for anaphylaxis, which indicates high allergenic potential *in vivo*.[10–13] A novel approach to creating a hypoallergenic preparation of Ara h 2 and Ara h 6 involves chemical modification that results in low IgE binding and preserved immunogenicity.[14] These chemical modifications reduced the IgE-binding ~100-fold in solid-phase immunoassays without reducing T-cell immunogenicity.[15]

An important question is whether the reduction of IgE-binding observed in solid-phase IgE-binding assays, such as the UniCAP system (Thermo Fisher Scientific, Portage, MI) and enzyme-linked immunosorbent assay, is functionally relevant. We sought to investigate the allergenicity of native and chemically modified CPE and the purified mix of Ara h 2 and Ara h 6 (Ara h 2/6) by using the *in vitro* mediator-release assay and passively sensitized with IgE antibodies from individuals with peanut allergy. The rat baso-

Table 1. **Characteristics of 26 subjects with peanut reaction**

	Age (y)	Sex	Peanut-sIgE (kU_A/L)	rAra h 2-sIgE (kU_A/L)	Total IgE (kU_A/L)	Peanut/Total IgE	rAra h 2/Total IgE	History of Reactions to Peanut
Responder No.*								
1	2	m	71.4	136	251	0.28	0.54	Urticaria, wheezing, emesis
2	2.5	m	40.3	47.4	97	0.42	0.49	Cough, wheezing, pruritus
3	3.5	f	49.4	62.1	183	0.27	0.34	Facial urticaria and angioedema, erythema
4	3.5	f	1110	65.4	275	4.0	0.24	Not exposed, always avoided based on high test results
5	4.5	m	1110	422	2244	0.49	0.19	Not exposed, always avoided based on high test results
6	5.5	f	307	93.6	553	0.56	0.17	Angioedema, urticaria, dyspnea, cough, rhinorrhea, emesis
7	5.5	m	252	186	599	0.42	0.31	Emesis, pruritus
8	5.5	m	342	159	1176	0.29	0.14	Not exposed, always avoided based on high test results
9	6	f	59.3	54.5	272	0.22	0.20	Urticaria, angioedema, flushing, pharyngeal pruritus
10	6	m	74.1	84.8	274	0.27	0.31	Rhinorrhea
11	7	m	79	37.9	184	0.43	0.21	Urticaria, emesis, wheezing, abdominal pain, throat tightness, dyspnea
12	7	f	233	129	596	0.39	0.22	Pruritus
13	7.5	f	214	132	413	0.52	0.32	Angioedema, urticaria
14	8.2	m	377	243	813	0.46	0.30	Angioedema, dyspnea, emesis
15	10	m	1870	531	4925	0.38	0.11	Angioedema, urticaria, wheezing, emesis
16	10	m	339	158	625	0.54	0.25	Not exposed, always avoided based on high test results
17	10	m	64.9	60	389	0.17	0.15	Pruritus
18	11	m	60.1	35	515	0.12	0.07	Angioedema, dyspnea, urticaria, emesis
19	11	f	258	149	386	0.67	0.39	Angioedema, urticaria, erythema, dyspnea, cough, rhinorrhea, throat tightness
Median (IQR)	6 (4.8–9.6)		233 (66.5–341.3)	129 (60.5–158.8)	413 (272.5–618.5)	0.42 (0.27–0.51)	0.24 (0.17–0.32)	
Nonresponder No.								
20	3.5	m	137	39.8	3815	0.0	0.01	Not exposed, always avoided based on high test results
21	5.5	m	19.5	15.2	155	0.1	0.10	Urticaria, dyspnea
22	7	f	29	16.8	100	0.3	0.17	Facial erythema, angioedema of eyes and lips
23	13	m	20.8	21.6	608	0.0	0.04	Urticaria
24	13.5	m	20.2	16	88	0.2	0.18	Nasal congestion, cough, flushing
25	20	f	54.7	65	174	0.3	0.37	Oral pruritus
26	35	f	36.2	28.6	298	0.1	0.10	Oral pruritus, chest tightness, conjunctivitis, vomiting, erythema
Median (IQR)	13 (5.9–18.4)		29 (20.4–50.0)	21.6 (16.2–37)	174 (113.8–530.5)	0.13 (0.057–0.28)	0.10 (0.05–0.18)	
Responders vs nonresponders, *p* value	0.087#		0.002§	< 0.001§	0.165#	0.026§	0.034§	

Total IgE and specific IgE (sIgE) were measured (range, < 2 [undetectable] to > 5000 and < 0.35 kU_A/L [undetectable], respectively) by using the UniCAP system; we obtained specific IgE from the sera > 100 kU_A/L by diluting 100 times in the sample diluent.

**Responders were defined as those whose sera produced maximum NHR ≥ 10% to CPE or native Ara h 2/6 at 1–10 ng/mL.*

#p = not statistically significant, §p < 0.05 was considered statistically significant (SigmaStat 3.5, Mann-Whitney rank sum t-test).

IQR = interquartile range.

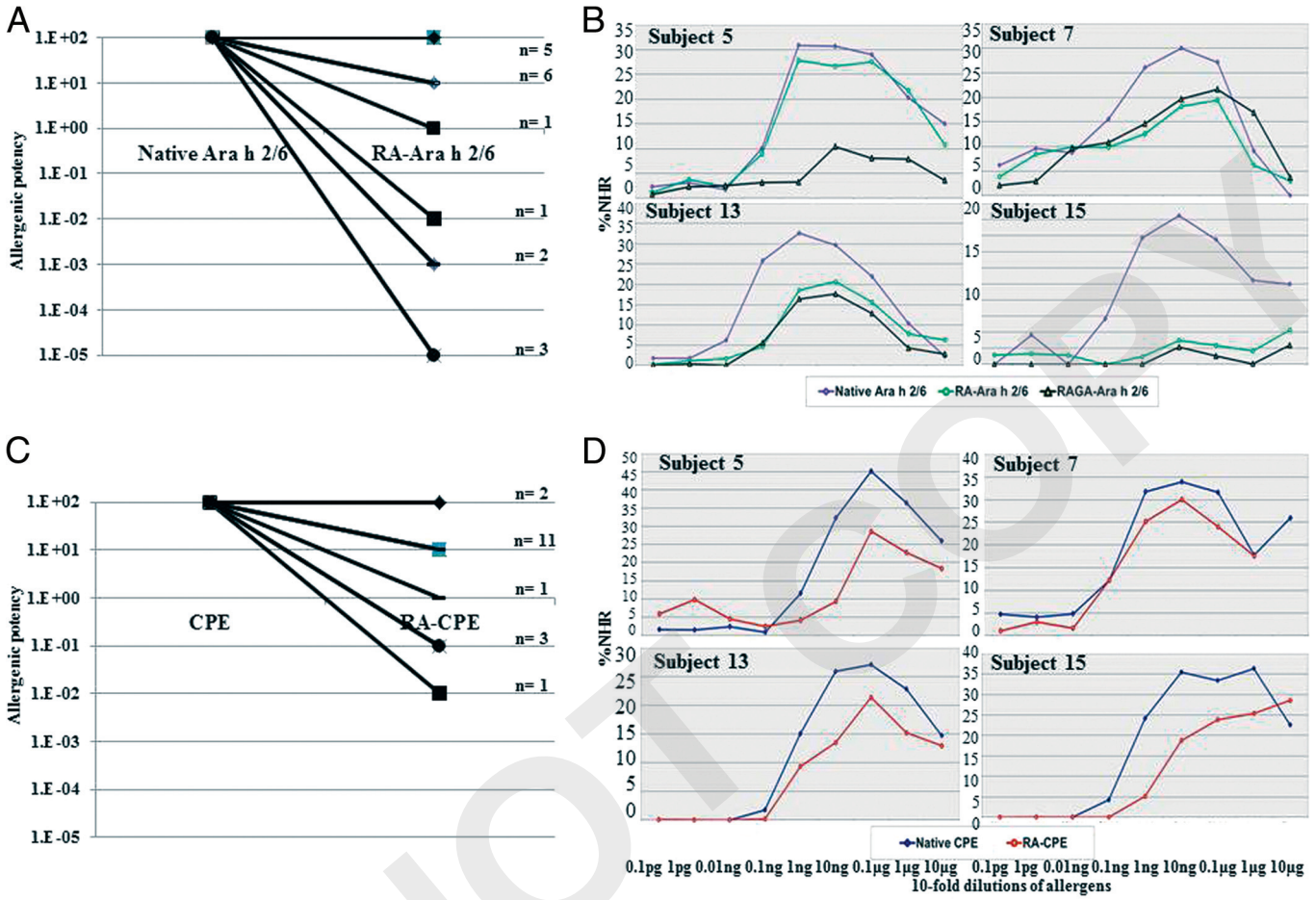

Figure 1. *(A and B) Allergenic potency and dose-response curve of NHR (%) between native Ara h 2/6 and its modified form, RA-Arah 2/6, and (C and D) CPE and its modified form. The dilution that gives the ED_{50} was calculated. The reciprocal value of ED_{50} (1/ED) was defined as the allergenic potency of the extract (1.E) and compared between native extract and its modified form on a logarithmic scale. Lines connect the symbols that represent the allergenic potency of native and RA-Ara h 2/6 extracts from the sera of the same individual subject. The overlapping number of subjects is shown. One responder (subject no. 12) was excluded because no NHR was induced by the RA-Ara h 2/6 extract. Allergenic potency of RAGA-Ara h 2/6 extract was not shown due to the unreliability of the allergenic potency from very low NHR (%). (A) Allergenic potency of native Ara h 2/6 and its modified form, RA-Ara h 2/6 in 19 responders. (B) Dose-response curve of NHR induced by native Ara h 2/6 and its modified forms, RA-and RAGA-Ara h 2/6 in four representative responders. The curves represent NHR (%) from the lowest peanut protein concentration (0.1 pg/mL, left) to the highest protein concentration (10 µg/mL, right). (C) Allergenic potency of CPE and its modified form, RA-CPE in 19 responders. Lines connect the symbols that represent the allergenic potency of CPE and RA-CPE extracts from the sera of the same individual subject. The overlapping number of subjects was shown. One responder (subject no. 12) was excluded due to no NHR induced by the RA-CPE extract. (D) A dose-response curves of NHR induced by CPE and its modified form, RA-CPE in four representative responders; the curves represent NHR (%) from the lowest peanut protein concentration (0.1 pg/mL, left) to the highest protein concentration (10 µg/mL, right).*

philic leukemia (RBL) cell line transfected with human FcεRI receptor were used because of their documented high affinity of human IgE binding and the ability to detect allergens at very low concentrations, which might not be detected in less-sensitive biochemical and immunochemical assays.[16–20]

Furthermore, the mediator-release assay can be performed with sera selected for optimal performance in a wide range of protein concentrations and experimental batch testing, and can be stored frozen for prolonged periods of time, which results in more cost-effectiveness and less variability than in the basophil activation test based on the donor basophils.

Findings

Subjects

Sera were obtained from 26 subjects with a convincing history of peanut allergy (16 males; median age 7 years, 25–75% interquartile range, 5.5–10) (Table 1). Subjects were recruited from the pediatric allergy practice at the Jaffe Food Allergy Institute. The study was

approved by the Icahn School of Medicine at Mount Sinai Institutional Review Board, and informed consent was obtained.

MATERIALS AND METHODS

CPE was prepared from defatted peanut (Virginia variety) flour and was subsequently reduced and alkylated (RA-CPE).[14] CPE was prepared from defatted peanut flour (Virginia-type peanuts) and was subsequently reduced and alkylated (RA-CPE) by reducing the disulfide bonds and alkylating the resulting free cysteines. Ara h 2/6 was purified as published,[7] and two forms of chemically modified Ara h 2/6 were prepared (RA-Ara h 2/6, as described for RA-CPE), and reduction and alkylation in combination with additional cross-linking by glutaraldehyde (RAGA-Ara h 2/6).[14]

A mediator-release assay was performed as previously described.[19] Sera from the subjects with peanut allergy were used for an overnight passive sensitization of RBL cell lines. A serum pool made from equal parts of 10 individual sera of responders (sera: 1, 6, 7, 10, 11, 13, 15, 16, 17, and 19) (Table E1) with high peanut-, rAra h 1–, and rAra h 2–specific IgE antibody levels (mean, 352.9, 149.1, and 156.8 kU_A/L, respectively) was used to optimize the peanut protein concentration range and serum dilution (1:20 and 1:40). Thereafter, RBL cells were stimulated with allergenic extracts at 10-fold dilutions from 0.1 pg/mL to 10 μg/mL and with serum dilution at 1:40 in triplicates. The extracts were used without a freeze-thaw cycle more than twice to avoid proteins refolding. Peanut allergen–induced NHR in the supernatant was used as a marker of RBL degranulation. Rabbit IgG antihuman polyclonal IgE (Bethyl Laboratories, Inc, Montgomery, TX) was used as a positive control.[13] Results were expressed as the percentage of release from cells sensitized with individual serum minus spontaneous release (with buffer), which was then divided by total release with 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) as follows:

% NHR = ([release by allergen – spontaneous release]/release by Triton X) × 100%

The dilution that gave the half maximal release was calculated (ED_{50}). The reciprocal value of ED_{50} (1/ED) was defined as the allergenic potency of the extract compared between native peanut extract and its modified form.

Nineteen responders were arbitrarily defined as those whose sera produced maximum NHR ≥ 10% to CPE or native Ara h 2/6 at 1–10 ng/mL concentration. Peanut-, rAra h 2-specific IgE (sIgE), rAra h 2-sIgE/total IgE ratio, and peanut-sIgE/total IgE ratio measured by UniCAP system were significantly higher in responders than in nonresponders (Table 1).

Table 2. **Allergenic potency reduction of chemically modified RA-Ara h 2/6 extract**

Responder No.	Potency of Native Ara h 2/6	Potency of RA-Ara h 2/6 Relative to the Potency of Native Ara H 2/6
1	100	10
2	100	1
3	100	100
4	100	100
5	100	0.01
6	100	100
7	100	10
8	100	10
9	100	10
10	100	0.001
11	100	100
12	100	0
13	100	0.00001
14	100	0.00001
15	100	0.00001
16	100	10
17	100	10
18	100	0.001
19	100	100

Table 3. **Allergenic potency reduction of chemically modified RA-CPE extract**

Responder No.	Potency of Native CPE	Potency of RA-CPE Relative to the Potency of Native CPE
1	100	100
2	100	10
3	100	10
4	100	10
5	100	0.1
6	100	10
7	100	10
8	100	10
9	100	10
10	100	10
11	100	10
12	100	0
13	100	0.01
14	100	0.1
15	100	0.1
16	100	10
17	100	10
18	100	1
19	100	100

Table 4. **Peanut allergen-induced NHR with 19 responder sera**

Responders, $n = 19$	Native Peanut Extract NHR, % (median 25–75% IQR)	Modified Peanut Extract NHR, % (median 25–75% IQR)		p Value
At the maximum release				
CPE	15.1 (7.2–26.8)	RA-CPE	12.2 (3.5–24.2)	0.189*
Ara h 2/6	18.5 (11.5–31.9)	RA-Ara h 2/6	5.2 (2.2–19.3)	0.001
		RAGA-Ara h 2/6	9 (2.7–16.5)	0.001
At 0.1 ng/mL				
CPE	1.7 (0.2–2.5)	RA-CPE	0.2 (0–1)	0.079*
Ara h 2/6	10.9 (8.2–17.5)	RA-Ara h 2/6	0.7 (0–3.9)	< 0.001#
		RAGA-Ara h 2/6	2.4 (0.6–4.4)	< 0.001#
At 1 ng/mL				
CPE	7.6 (2.6–12.9)	RA-CPE	0.8 (0.2–4.0)	< 0.001#
Ara h 2/6	15.7 (9.3–31.9)	RA-Ara h 2/6	1.2 (0.4–9.0)	< 0.001#
		RAGA-Ara h 2/6	3.5 (0.7–9.0)	< 0.001#
At 10 ng/mL				
CPE	15.1 (7.2–25.9)	RA-CPE	3 (1–11.4)	0.002
Ara h 2/6	13.9 (3.5–28.6)	RA-Ara h 2/6	4.4 (1–17.8)	0.026
		RAGA-Ara h 2/6	4.5 (2.1–13.1)	0.041

**p = not statistically significant, #p < 0.05 was considered statistically significant, (SigmaStat 3.5, Mann-Whitney rank sum t-test).*
IQR = interquartile range.

RESULTS

The calculated allergenic potency and NHR dose-response curve of native extract and its modified form are shown in Fig. 1. Fig. 1, *A* and *B* represent native Ara h 2/6 and RA-Ara h 2/6; Fig. 1, *C* and *D* represent CPE and RA-CPE. NHR induced by chemically modified extracts was reduced compared with their native counterparts. (Tables 2 and 3) Chemical modification resulted in an onset of activation at a higher allergen concentration by 10-to 100-fold as well as in a lowering of the maximum NHR. Modified RA-Ara h 2/6 and RAGA-Ara h 2/6 caused significantly lower maximum mediator release than native Ara h 2/6 at protein concentration 0.1, 1, and 10 ng/mL ($p < 0.001$, $p < 0.001$, $p < 0.001$, respectively, for RA; and at $p < 0.001$, 0.026, and 0.041, respectively, for RAGA) (Table 4). RA-CPE caused significantly lower maximum NHR than native CPE at protein concentrations 1 ng/mL ($p < 0.001$) and 10 ng/mL ($p < 0.002$) (Table 4). There was a significant positive correlation between rAra h 2-specific IgE and the maximum NHR induced by native and chemically modified peanut extracts (Table 5). Responders had high rAra h 2 IgE (mean, 61.1 kU_A/L; $p < 0.001$) and had higher NHR in mediator release assay to native Ara h 2/6 than CPE (data not shown), which indicates that Ara h 2/6 was the most relevant peanut allergen in these responders. IgE antibody levels and mediator release were positively correlated and associated with responder status.[19] We observed a similar association (Table 5).

DISCUSSION

We demonstrated that chemical modification of peanut resulted in ~100-fold reduction in mediator release in an *in vitro* assay, which indicates a significantly decreased allergenicity. This is in line with observations made for modified Ara h 2/6 when using a solid-phase IgE-binding assay and findings of a recent study performed in European adult subjects.[15]

We focused on conglutin storage Ara h 2 due to its high allergenic potency and resistance to digestive proteases pepsin and trypsin. In addition, Ara h 6 has a high homology of amino acid sequence to Ara h 2, especially in the middle part and at the C-terminal part of the protein from peanut. Koppelman *et al.*[21] showed the cross-reactivity in IgE binding of purified Ara h 6 and Ara h 2 described as potent allergens in peanut. Vissers *et al.*[22] reported that heat-induced conformation of native Ara h 2/6 purified after roasting retained its native forms and that extensively heat-induced denaturation did not affect the allergenicity properties of Ara h 2/6 from roasted peanut. Thus, a chemical protein modification strategy could be used as an alternative approach to destroy allergenic peptide epitopes with maintained immunogenicity.

Table 5. **Peanut allergen-induced NHR; 10 responders with high specific IgE toward rAra h 2 and low specific IgE against rAra h 1 <35 kU_A/L***

	CPE NHR, % (median 25–75% IQR)	Native Ara h2/6 Extract NHR, % (median 25–75% IQR)	*p* Value
At the maximum release			0.045
CPE	7.3 (5.6–12.4)		
Ara h 2/6		16.4 (10.8–25.4)	
At 0.01 ng/mL			0.005
CPE	0.4 (0.05–1.6)		
Ara h 2/6		8.4 (2.6–9.7)	
At 0.1 ng/mL			< 0.001#
CPE	0.6 (0–1.7)		
Ara h 2/6		10.8 (9–14.6)	
At 1 ng/mL			0.005
CPE	2.7 (2–7.3)		
Ara h 2/6		13.2 (7.8–22.4)	

**Specific IgE toward rAra h 1 (median, 17.5; median 25–75% interquartile range, 15.3–17.5) was significantly lower ($p < 0.001$) than specific IgE toward rAra h 2 (median, 61.1; median 25–75% interquartile range, 47.4–84.8).*
#$p < 0.05$ was considered statistically significant; NS = not statistically significant (SigmaStat 3.5, Mann-Whitney rank sum t-test).
IQR = interquartile range.

We found that chemical modification of crude peanut and purified native Ara h 2/6 reduced mediator release in an *in vitro* mediator-release assay ~100-fold, which indicates decreased allergenicity. Furthermore, it can be performed with sera selected for optimal performance in a wide range of protein concentrations and experimental batch testing, and can be stored frozen for prolonged periods of time, which results in more cost-effectiveness and less variability of basophil activation test from donors. We observed that some nonresponder sera with high-specific IgE to peanut and rAra h 2 induced a low mediator release, NHR < 10%. This low release might be explained by lower IgE antibody affinity, a lower number of recognized IgE-binding epitopes, or dilution effect, in the presence of high total IgE antibodies.

CONCLUSIONS

The confirmation of the decreased IgE binding of chemically modified native peanut proteins is an important step for the further development of the alternative candidate for safe and successful peanut immunotherapy. However, the wide range of the individual responses to chemically modified peanut proteins warrants caution and indicates that, before immunotherapy with chemically modified peanut proteins, careful patient characterization and selection must be considered.

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