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Comparison of cockroach allergenic activity in whole body and fecal extracts

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Previous studies have established cockroach allergens as important sensitizing agents in the induction/exacerbation of urban asthma. The present investigation compared saline extracts of American cockroach (Periplaneta americana) whole bodies and feces and German cockroach (Blattella germanica) whole bodies and feces as important sources of allergens. All extracts were tested before or after gel filtration on Sephadex G-75 columns (fraction 2) as previously described. Skin test studies of 69 subjects with asthma with extracts of American or German cockroaches demonstrated a significant correlation of reactivity to whole body and fecal extracts for both species. Direct RASTs of 13 sera from cockroach skin test-positive subjects were generally greater to both German whole body extracts (GWBEs) and German fecal extracts (GFEs) as compared to American whole body and fecal extracts. There was a good correlation of RAST reactivity to GWBE with GFE. RAST inhibition demonstrated that GFE contained most of the allergenic activity present in GWBE. These studies demonstrate the allergenic similarities of cockroach whole body and fecal extracts and suggest that cockroach feces are an important sensitizing agent in atopic asthma. (J ALLERGY CLIN IMMUNOL 1991;87:574-80.)

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Abbreviations used

AWBE:	American whole body extract
GWBE:	German whole body extract
AFE:	American fecal extract
GFE:	German fecal extract
Fr 2:	Fraction 2 of Sephadex G-75 column eluate containing enhanced cockroach allergenic activity
ID ₅₀ :	Inhibitory dose that would inhibit 50% of the RAST
IEF:	Isoelectric focusing
pI:	Isoelectric point

Previous studies established that cockroach allergens are important sensitizing agents in the induction/exacerbation of urban asthma.¹⁻⁷ In recent studies from our laboratory, approximately 50% of atopic, predominantly inner city residents had immediate wheal-and-flare cutaneous reactivity to commercial cockroach whole body extracts.⁸ Further studies with whole body extracts prepared in our laboratory from both American (*Periplaneta americana*) and German (*Blattella germanica*) cockroaches confirmed these findings.⁹ In spite of significant subject reactivity to cockroach extracts, the specific source of the allergen remains unclear. Clearly, body parts, exoskeleton, secretions, and egg casings are disseminated in house dust and may be sources of allergen. Fecal particles and/or their fragments can also be present in dust.

In a recent study performed in our laboratory, 63% (29/46) of atopic subjects with asthma demonstrated significant skin test reactivity to both fecal and whole body extracts from American and German cockroaches.⁹ The following study was undertaken to assess further the reactivity of cockroach-sensitive subjects to fecal allergens and the relationship of these allergens to allergens in cockroach whole body extracts.

MATERIAL AND METHODS

Study subjects

Sixty-nine atopic subjects with asthma, presenting to our allergy clinics at Tulane University School of Medicine, Charity Hospital of New Orleans, and the Louisiana State University Medical Center, Shreveport, La., were chosen for study. Informed consent was obtained, and all subjects were skin prick tested with AWBE, AFE, GWBE, and GFE prepared, as described below, at 10 mg/ml in 50% glycerol. Furthermore, an additional 13 subjects with asthma who were previously demonstrated to be skin test and RAST positive to cockroach extracts were bled for sera for RAST and RAST-inhibition studies.

Cockroach extracts

American or German cockroaches of both sexes (obtained from the Carolina Biological Supply Co., Burlington, N.C.)

were received in the shipping crate and kept at room temperature for 5 to 7 days. Pieces of apple were added to each crate to provide food and moisture for the insects. Cockroaches were killed by freezing at -70°C . Cockroach whole bodies and fecal pellets were identified macroscopically and removed separately with forceps. Whole body extracts were prepared by homogenizing cockroaches in saline with a Waring blender (Waring Products Div., New Hartford, Conn.) (three 1-minute pulses). The homogenate was mixed overnight at 4°C and centrifuged (18,000 g), and the supernate was concentrated (Amicon [Amicon Corp., Danvers, Mass.] YM2-MWCO, 1000 daltons). The concentrate was centrifuged (105,000 g), dialyzed (MWCO, 3500 daltons) against saline, and aliquoted. Dry weights were determined, and aliquots were frozen at -20°C . Feces were extracted in a similar manner. Cockroach whole body and fecal extracts were separately fractionated on a Sephadex G-75 column. Column fractions were pooled and tested for allergen activity by RAST inhibition.

RAST and RAST inhibition

Direct RAST was performed in duplicate with cockroach antigen-coated paper disks (1 μg per disk) for the detection of serum IgE antibodies specific for cockroach allergens as described previously.⁸ Results were expressed as the percent ^{125}I -labeled anti-IgE bound, obtained by dividing the mean counts per minute of the test sample by the total counts per minute of the ^{125}I -labeled anti-IgE added to the reaction (approximate 25,000 cpm) $\times 100$. For RAST inhibition, 50 μl of inhibitor was incubated with 50 μl of sera pool diluted 1:10 (composed of equal volumes of nine sera) in the presence of cockroach allergen-coated disks overnight on a rotator at 24°C . Disks were then washed, and ^{125}I -labeled anti-IgE was added and incubated overnight. Disks were washed again, and the remaining activity was counted in a gamma counter. Results were expressed as the percent inhibition of radiolabeled anti-IgE bound as compared to a control reaction in which phosphate-buffered saline was added instead of inhibitor.

The correlation of values for whole body and fecal RAST was determined by Spearman's rank correlation. Slope values of the linear ascending portion of the RAST-inhibition curves were compared by linear regression. Comparison indicates qualitative (different slope with common intercept) and/or quantitative (common slopes with different intercept) differences between the solid phase and inhibitor.¹⁰

To estimate biologic activity, ID₅₀ was estimated graphically by plotting the different dilutions of test material against their RAST-inhibition activity. The total activity (number of ID₅₀ values) within a particular sample was calculated by dividing the total sample (either weight or volume) by its ID₅₀ (expressed in weight or volume).

IEF

Previous studies demonstrated that all proteins within gel filtration Fr 2 of AWBE and GWBE had pIs below pH 7.5 when proteins were focused on polyacrylamide gels. Thus, all additional studies used 5% polyacrylamide gel plates (Ampholine PAG plate, Pharmacia/LKB, Bromma, Sweden) with an ampholyte range of pH 4.0 to 6.5. IEF of

TABLE I. Correlation of skin test reactivity to GWBE with GFE

	GFE skin tests		
	+	-	Total
GWBE skin tests			
+	37	4	41
-	0	28	28
Total	37	32	69

$p = 2.14 \times 10^{-15}$ by Fisher's exact test.

GWBE, GWBE Fr 2, GFE, and GFE Fr 2 was performed as previously described¹¹; gels were fixed and stained with 0.1% Coomassie brilliant blue R-250.

RESULTS

Skin test reactivity

Significant skin test reactivity was observed to whole body and fecal extracts of American and German cockroaches (Tables I and II). A comparison of skin test reactivity of whole body to fecal extracts for each cockroach species demonstrated a significant correlation for GWBE to GFE ($p = 2.14 \times 10^{-15}$; Table I) and AWBE to AFE ($p = 1.01 \times 10^{-11}$; Table II).

Fractionation of GWBE and GFE by gel filtration

Cockroach whole body and fecal extracts were fractionated on a Sephadex G-75 column, and fractions were analyzed for ultraviolet absorbing activity. Fractions were pooled according to their ultraviolet absorbance, and allergenic activity was determined by RAST inhibition. The results summarized in Figs. 1 and 2 demonstrate that most allergenic activity was present in the column eluate that was included in the column to the cytochrome c marker (Fr 2) for both the fecal and whole body extracts.

The recovery of cockroach extract (milligrams) and allergenic activity (expressed as ID₅₀ in micrograms) is summarized in Table III after fractionation of GWBE and GFE on Sephadex G-75 column. These results indicate that, although activity was present in Frs 1 to 3 for both extracts, clearly Fr 2 contained maximal activity. Fr 2 accounts for 28.6% and 26.7% of the activity and weight recovery of GWBE, respectively, and 24.0% and 24.9% of the activity and weight recovery of GFE, respectively. This represents more than two thirds of the activity recovered from either fractionation. Clearly, Fr 2 is the most potent fraction from both GWBE and GFE and was used for additional testing and analyses.

TABLE II. Correlation of skin test reactivity to AWBE with AFE

	AFE skin tests		
	+	-	Total
AWBE skin tests			
+	34	9	43
-	0	26	26
Total	34	35	69

$p = 1.01 \times 10^{-11}$ by Fisher's exact test.

IEF

IEF followed by Coomassie brilliant blue staining demonstrated that both GWBE and GFE extracts had more precise patterns of protein banding after gel filtration, demonstrating that each contained reduced amounts of nonfocusing material (Fig. 3). Clearly, the number and intensity of protein bands increased after gel filtration. Twenty-six protein bands were resolved in GWBE Fr 2 (pI, 3.5 to 6.9) and 14 protein bands in GFE Fr 2. The position (pI) of 11/14 GFE Fr 2 bands corresponded to bands present in GWBE Fr 2.

Correlation of GWBE and GFE RASTs

RAST results to GFE Fr 2 and GWBE Fr 2 for individual sera were plotted as illustrated in Fig. 4. The Spearman's rank-correlation value between GWBE and GFE RASTs ($r = 0.742$; $p < 0.01$) demonstrated a correlation of RAST reactivity. Direct RASTs of individual sera of cockroach skin test-positive subjects were generally greater to both GWBE and GFE as compared to AWBE and AFE.

Inhibition of GWBE and GFE RASTs by GWBE and GFE

RAST-inhibition studies were performed to inhibit GWBE RAST with GWBE and GFE and GFE RAST with GWBE and GFE (Fig. 5). The results (representative of three separate experiments with three sets of extracts) demonstrate that significant inhibition of both RASTs was obtained with both cockroach extracts. Statistical analysis indicated no significant difference between the inhibition slopes within either set of inhibitions. This indicates allergenic similarity of the two sources within each inhibition. However, although this finding was not statistically significant, a consistently greater inhibition was obtained in each case with homologous as compared to heterologous extracts.

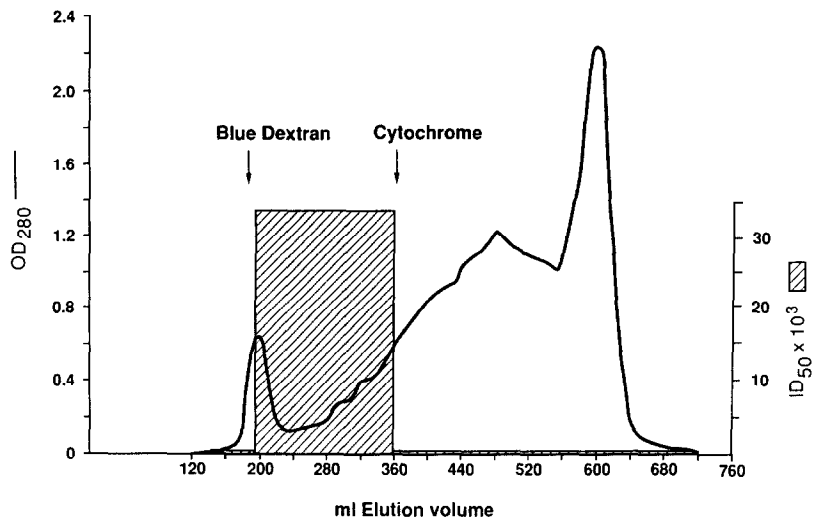


FIG. 1. Gel filtration of GWBE on Sephadex G-75 column; 55.5 mg of GWBE was loaded on a 100 by 2.6 cm column (bed volume, 530 cc) and eluted with phosphate-buffered saline. Column fractions (4 ml) were collected dropwise and analyzed for ultraviolet-absorbing material by spectroscopy, and for cockroach allergenic activity by RAST inhibition.

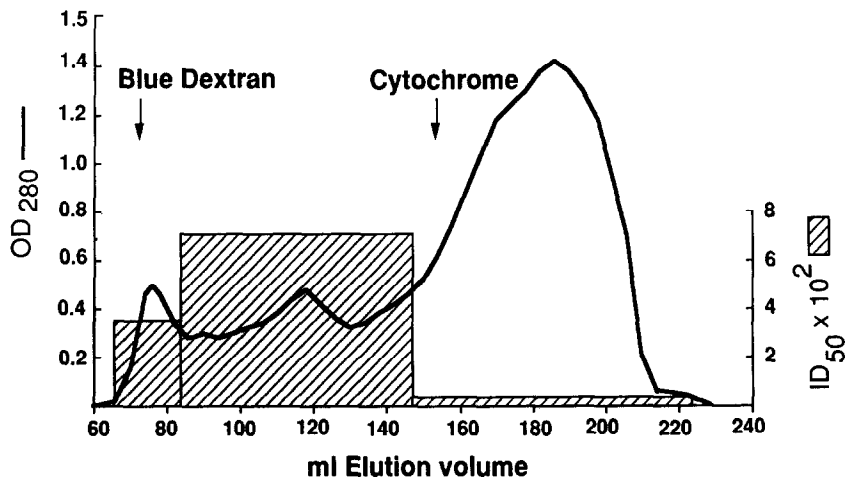


FIG. 2. Gel filtration of GFE on Sephadex G-75 column. GFE (43.5 mg) was loaded 1.6 cm column (bed volume, 201 cc), and 2 ml fractions were collected and analyzed, in Fig. 1.

TABLE III. Fractionation of cockroach extracts

Sample	Total material (mg)	ID ₅₀ (μg)	Total activity ID ₅₀	% Recovered	
				ID ₅₀	Weight
GWBE	55.50	4.6	12,065	100.0	100.0
GWBE Fr 1	3.00	140.0*	21	0.1	5.4
GWBE Fr 2	14.85	43.0	3,453	28.6	26.7
GWBE Fr 3	32.90	370.0	88	0.7	59.2
GFE	43.50	14.0	3,107	100.0	100.0
GFE Fr 1	7.00	21.5	325	10.5	16.1
GFE Fr 2	10.80	14.5	745	24.0	24.9
GFE Fr 3	19.40	500.0*	38	1.2	44.6

*Estimated by extrapolation.

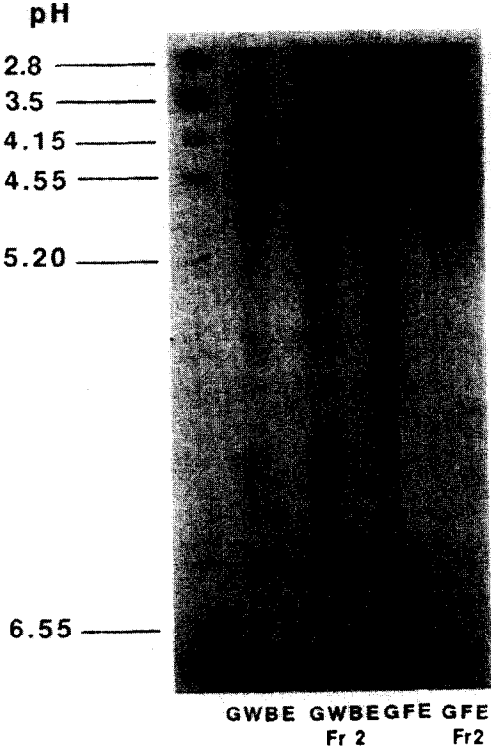


FIG. 3. Analysis of German cockroach extracts by IEF. Crude and partially purified cockroach extracts were separated by analytical IEF on PAG plates, and proteins were fixed and stained with Coomassie brilliant blue.

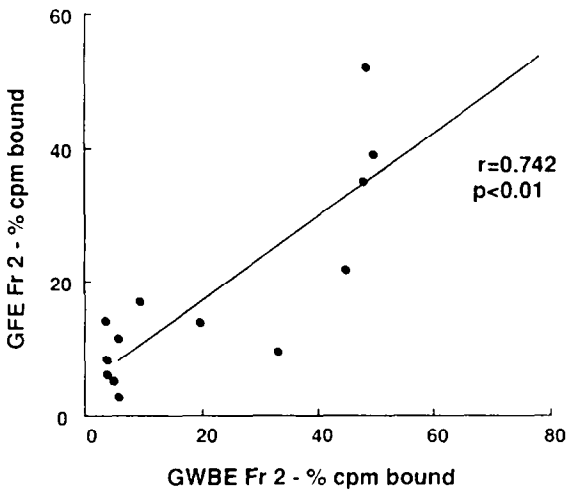


FIG. 4. Correlation of RAST activity to GWBE Fr 2 with GFE Fr 2. RAST activity for individual sera to GWBE and GFE were plotted and analyzed by Spearman's rank correlation. Results were highly correlated ($r = 0.742$; $p < 0.01$).

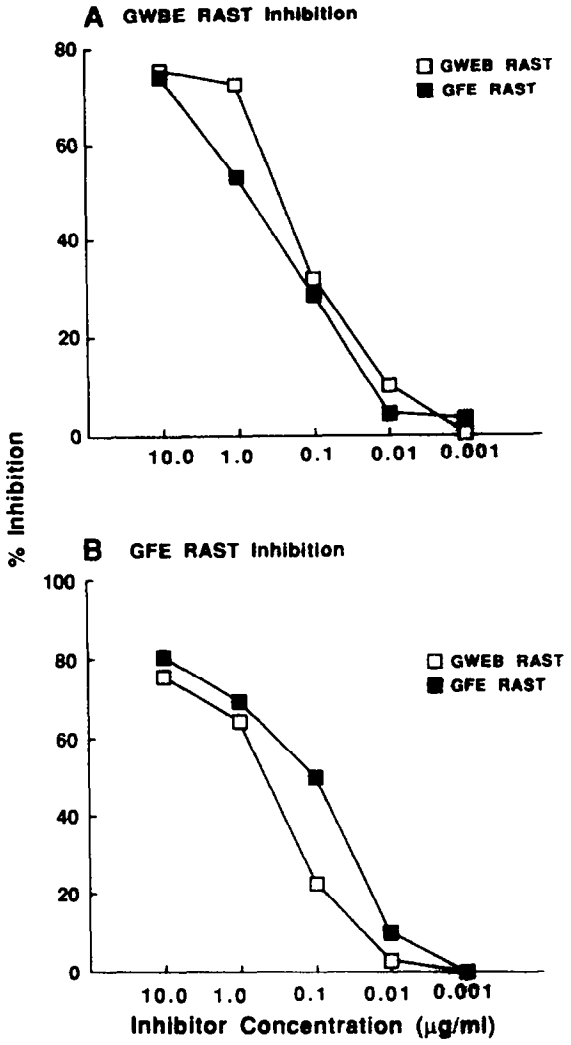


FIG. 5. Inhibition of GWBE and GFE RASTs with GWBE and GFE extracts. Increasing concentrations of cockroach extracts were used to inhibit cockroach RASTs. Results demonstrate a high degree of similarity among the allergenic components of both preparations.

DISCUSSION

Previous studies stated that cockroaches were important components of house dust allergens.¹²⁻¹⁵ During the last 10 years, a number of studies have strongly suggested that cockroach allergens are a significant cause of asthma, particularly among patients living in large urban areas in the United States.^{3, 5, 16, 17} These studies indicate that a high percentage of patients with asthma have significant skin test reactivity and serum IgE antibodies to cockroach allergens and exhibit positive bronchoconstrictive responses on exposure to cockroach extracts.^{3, 5, 16, 17} Thus, it is clear that, where

cockroach allergen is present in sufficient quantities, it is a major indoor air allergen.

During infestation of a particular house, cockroaches can produce a variety of materials that may have potential allergenic consequence. Thus, secretions, egg casings, body parts, as well as excrement, all can be incorporated into dust and could contribute to cockroach allergenic activity. Bernton and Brown¹⁸ presented skin test results that suggested the presence of allergens in German cockroach feces. These studies were supported by observations of Choovivathanavanich¹⁹ in Thailand who demonstrated that 52% of 163 allergic patients randomly selected for study gave positive skin responses to both whole body and fecal cockroach extracts. These investigators concluded that both cockroach fragments and feces in house dust can be an important source of allergens. A contradictory study by Richman et al.²⁰ investigated several sources of cockroach allergens from American and German cockroach whole bodies, cast skins, and feces, as well as egg casings of German cockroach. Their results indicated that, for German cockroach, whole body and cast skins have significant RAST potency, whereas egg casings were less potent and feces were the least potent.

Recently, Menon et al.⁹ investigated 46 subjects with asthma attending the Louisiana State University Hospital Allergy Clinic at Shreveport for skin test reactivity to a number of cockroach extracts. Significant skin test reactivity was demonstrated to extracts of American and German cockroach whole bodies, as well as fecal extracts from American and German cockroaches. Those studies were extended in the current study in which we compared skin test reactivity of 69 patients to AWBE with that to AFE, as well as GWBE to GFE. The results demonstrate a high correlation of skin test reactivity to whole body and fecal extracts for both species. Clearly, it is possible that this reactivity may reflect a parallel sensitization to these different sources of cockroach allergens. However, our present RAST-inhibition results suggest that it is due to the presence of shared allergens. Although there is significant cross-reactivity of whole body and fecal allergens, the fact that 4/41 patients who were GWBE positive were skin test negative with GFE and that 9/43 patients who were AWBE positive were negative with AFE suggests that whole body extracts may contain unique allergens or allergens in concentrations different from concentrations in fecal extracts.

The present study demonstrates a positive correlation between RAST reactivity to whole body and fecal extracts, as well as significant inhibition of GWBE and GFE RASTs by GWBE and GFE. We

conclude from these results that allergenic composition of whole body and fecal extracts is quite similar. However, because these results were based on investigations with pooled sera, and GWBE and AWBE were more sensitive than GFE and AFE, respectively, qualitative or quantitative differences may exist for individual subjects. Furthermore, gel filtration chromatographic studies of GWBE and GFE indicate similar elution properties of allergens on Sephadex G-75 columns, indicating that the allergens from these two sources cover a similar molecular weight range. Our IEF results further demonstrate that many proteins in both sources have similar pI values.

Horner et al.²¹ have taken these studies one step further by comparing extracts of GWBE and GFE by immunoprint and immunoprint-inhibition assays. Their studies demonstrate a remarkable similarity of fecal and whole body allergen extracts and suggest that most of the allergens cross-react. Thus, based on these observations, we conclude that fecal material from cockroaches is an important source of cockroach allergens. In fact, it is highly probable that fecal material may be a more important source of cockroach allergens than chitinous body parts, since desiccated cockroach excrement is more likely to crumble and become airborne as disseminated dust. These findings have serious implications for individuals living in homes with covert and hence unsuspected cockroach infestations, since fecal material may not be detected as easily as live cockroaches, whole bodies, or body parts. Thus, homes that are covertly infested, that is, infestations in which roaches and/or bodies are not frequently observed, may still contain appreciable amounts of fecal material because of roach activity, even if cockroaches are not a recognized problem.

The question of the primary origin of cockroach allergens remains. Do whole body cockroach extracts contain allergenic activity that merely reflects residual feces present in these insects? Alternatively, is it possible that allergenic activity in cockroach excrement reflects secretions from whole body that are present in the feces? One approach may be to prepare extracts of cockroach whole body from which the intestine has been removed and to test these extracts in sensitive patients. Alternative approaches will be to identify major cockroach allergens and then assess their presence in whole body or fecal material.

It is of interest that our studies demonstrate significant RAST reactivity to both GWBE and GFE, whereas studies by Richman et al.,²⁰ although they reported significant reactivity to GWBE, detected little RAST reactivity to fecal extracts. The difference in these results may be explained by differences in

extraction methods. Although our extractions were routinely performed at 4° C overnight, extractions of Richman et al. were performed at 23° C for 24 hours. Generally, fecal material contains a high concentration of proteolytic enzymes, and it is quite possible that during the extraction process at 23° C, much of the allergenic activity present in feces was destroyed.

Although these studies have demonstrated that allergens are present in feces, the nature of cockroach fecal allergens remains undetermined. In spite of the remarkable similarity between fecal and whole body allergen content, unique allergens may be present in feces as well as whole bodies. This is due to the fact that our inhibitions of GWBE or GFE RASTs were maximal with homologous rather than heterologous extracts. Furthermore, immunoprint inhibition was always greatest with homologous rather than heterologous inhibitors.²¹ The identification of any unique allergens present in whole body or feces, however, awaits isolation and identification of individual cockroach allergens. The recent studies of Lind et al.²² and Helm et al.,²³ as well as studies of Pollart et al.,²⁴ identifying and isolating cockroach allergens through chromatographic methods and immunoabsorbent techniques, will help provide reagents with which to further analyze shared and unique allergens present in cockroach whole bodies and feces.

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