INFECTION AND IMMUNITY, Sept. 1986, p. 651–655
0019-9567/86/090651-05$02.00/0

Vol. 53, No. 3

Late Intraphagosomal Hydrogen Ion Concentration Favors the In Vitro Antimicrobial Capacity of a 37-Kilodalton Cationic Granule Protein of Human Neutrophil Granulocytes

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Received 18 February 1986/Accepted 16 May 1986

We described previously (W. M. Shafer, L. E. Martin, and J. K. Spitznagel, Infect. Immun. 45:29–35, 1984) the presence of a 37-kilodalton cationic antimicrobial protein (37K CAP) in extracts of granules prepared from human polymorphonuclear granulocytes (PMN). In this investigation, we prepared 37K CAP from PMN granule extracts by sequential ion-exchange and molecular-sieve chromatography and examined its antimicrobial activity against a number of gram-negative and gram-positive bacteria. At concentrations of 5 μg/ml or lower, 37K CAP exerted selective antimicrobial activity against gram-negative bacteria. These bacteria included *Acinetobacter lwoffii*, *Escherichia coli*, *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, *Pseudomonas cepacia*, *Salmonella typhi*, *Salmonella typhimurium*, and *Shigella sonnei*. However, at 5 μg of 37K CAP per ml, *Proteus mirabilis*, *Proteus vulgaris*, and *Serratia marcescens* resisted this antimicrobial activity. The bactericidal activity of 37K CAP was greatest in acidic (pH 5.5) as opposed to alkaline (pH 7.5) media. The level of *S. typhimurium* resistance to 37K CAP correlated with the presence of O antigen in the lipopolysaccharide. In the absence of O antigen repeat units, resistance was proportional to the length of the core oligosaccharide. These results suggest that 37K CAP may contribute significantly to the ability of PMN to kill gram-negative bacteria by nonoxidative means, particularly as the maturing phagolysosome becomes acidified.

Shortly after engulfment by polymorphonuclear granulocytes (PMN), bacteria become coated by a number of cationic proteins (11). These cationic proteins, derived from the cytoplasmic granules of PMN, are candidates for killing bacteria within PMN by nonoxidative processes (10, 12). The intimate association of these cationic proteins with the surface of bacteria suggests that the proteins with antimicrobial capacity must first bind to appropriate receptors in order to kill target bacteria. Our studies (8; H. Wollenweber, W. M. Shafer, J. K. Spitznagel, and D. C. Morrison, submitted for publication) with gram-negative bacteria have shown that two highly purified cationic antimicrobial proteins (CAPs) bind bacterial lipopolysaccharides (LPSs) with a high degree of specificity and affinity. Binding of CAPs was shown to occur within the lipid A or inner core region of the LPS.

The capacity of CAPs to kill bacteria within phagolysosomes may be regulated by the intraphagosomal hydrogen ion concentration. It has been generally appreciated that the intraphagosomal hydrogen ion concentration in time extends over a broad range, eventually becoming quite acidic (7). Thus, it would be advantageous to the O_2-independent antimicrobial arsenal to have components, such as CAPs, capable of bactericidal activity at various intraphagosomal hydrogen ion concentrations. Evidence presented here shows the existence of at least one granule-associated CAP (37K CAP) that functions in vitro over a pH range similar to that of maturing phagolysosomes and is most active at or near pH 5.5. This pH is considered to be at or near the final pH of the mature phagolysosome (7). These results suggest that 37K CAP may contribute significantly to the capacity of PMN to kill intraleukocytic bacteria by nonoxidative processes as the pH of the maturing phagolysosome declines after phagocytosis.

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MATERIALS AND METHODS

Preparation of PMN granule extracts and purification of 37K CAP. Granulocyte concentrates (>95% PMN) were obtained by leukapheresis from two healthy donors who had given informed consent at Emory University Hospital. The preparation and homogenization of PMN have been described previously (5). Granules, obtained by high-speed centrifugation (5), were extracted three times in 0.2 M sodium acetate (pH 4.0). Such extracts were stored at 4°C before use. We obtained 37K CAP as described by Shafer et al. (8). Briefly, a crude granule extract containing 125 mg of protein was dialyzed against 50 mM sodium acetate (pH 5.0) and 0.15 M NaCl at 4°C overnight and then applied to a carboxymethyl Sephadex (Pharmacia Fine Chemicals, Piscataway, N.J.) column (2.5 by 25 cm). Bound protein was eluted with a linear salt gradient, as described by Shafer et al. (8), and modified as described by Casey et al. (2). The 37K CAP emerged (as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]; see below) from the column at sodium chloride concentrations ranging from 0.6 to 0.7 M. This partially purified preparation of 37K CAP was dialyzed overnight at 4°C against 0.2 M sodium acetate (pH 4.0), concentrated by ultrafiltration (8), and applied to a Sephadex G-75(SF) column (0.25 by 50 cm). All protein concentrations were determined by the method of Bradford (1).

The purity of 37K CAP was analyzed by silver staining of SDS-PAGE (4) profiles, as described by Shafer et al. (8), and by analysis of its elution profile from a Waters C-18 reverse-phase high-performance liquid chromatography (RP-HPLC) column (see Fig. 2).

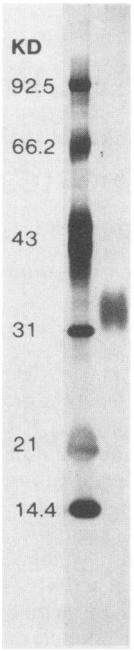

FIG. 1. Electrophoretic mobility of 37K CAP in SDS-PAGE. One microgram of 37K CAP was solubilized at 100°C for 5 min in 0.125 M Tris hydrochloride (pH 6.8) containing 2% (wt/vol) SDS and 1% (vol/vol) beta-mercaptoethanol. The solubilized protein was applied to a 15% SDS-PAGE gel containing a 4% stacking gel and subjected to electrophoresis at 35 mA. Protein was visualized by silver staining. The migration of 37K CAP was related to the molecular mass markers (14,400 to 92,500) as shown.

Bacteria studied and antimicrobial assay. The following strains of *Salmonella typhimurium* and their respective LPS chemotypes were used: LT2, smooth; SH 9013, Ra; SH 9178, Rb_1; SL-1004, Rd_1; SH 7518, Re; and SH 7519, Re but *pmrA* (13). We also examined the 37K CAP susceptibility of the following bacteria: *Acinetobacter lwoffii*, *Bacillus subtilis*, *Escherichia coli*, *Neisseria gonorrhoeae*, *Proteus mirabilis*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Pseudomonas cepacia*, *Salmonella typhi*, *Serratia marcescens*, *Shigella sonnei*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes*. All antimicrobial assays were performed as described by Rest et al. (6), except when gonococci were studied; in the latter case, the antimicrobial assay described by Casey et al. was used (2). Where indicated, the tryptone saline media used in the assays were prepared with different concentrations of NaCl or altered in pH as described by Rest et al. (6). Controls consisted of bacteria incubated in the absence of 37K CAP.

RESULTS

Purity of 37K CAP. Using the purification scheme described previously (8), we obtained 425 μg of 37K CAP from 125 mg of protein present in the crude granule extract obtained from ca. 10^{10} PMN. Thus, in good agreement with our earlier study, 37K CAP constituted ca. 0.34% of the total protein content of the crude extract. We found that in SDS-PAGE the 37K CAP preparation (1 μg of protein) migrated as a single but diffuse band (Fig. 1). As shown in Fig. 1, 37K CAP could be detected when the SDS-PAGE gel was stained with silver salts, although the staining intensity was substantially less than that of other granule proteins, e.g., cathepsin G and lysozyme (data not shown).

The purity of 37K CAP was evaluated further on the basis of its elution profile from a Waters C-18 RP-HPLC column. We found that when 5 μg of 37K CAP was applied to the column, the bound protein eluted from the column was a single peak, in typical gaussian fashion (Fig. 2).

Sensitivity of *S. typhimurium* to 37K CAP is dependent on temperature, pH, and ionic strength. Using the tryptone saline media described by Rest et al. (6), we found that 37K CAP exhibited potent antimicrobial activity against an Rd_1 LPS mutant (SL-1004) of strain LT2. We found that the ca. 50% effective dose was 5 μg of protein per ml (Fig. 3). At this concentration of 37K CAP, we found that antimicrobial activity was temperature dependent, since incubation of strain SL-1004 with 37K CAP at 4 or 22°C resulted in little or no killing, whereas substantial killing occurred at 37°C (data not shown).

We tested whether alterations in the pH or ionic strength of the tryptone saline media influenced the antimicrobial capacity of 37K CAP. We found that although 37K CAP exhibited antimicrobial activity over a broad range of pH (5.5 to 7.5), acidic conditions rendered strain SL-1004 less resistant to 37K CAP (Fig. 4). Conditions of ionic strength also appeared to be critical for maximal activity; the optimal ionic strength was found to be 37.5 mM NaCl (Fig. 4).

Once we had established the conditions necessary for optimal in vitro antimicrobial activity of 37K CAP, we examined a number of gram-positive and gram-negative bacteria for their susceptibilities to 37K CAP. Assays were performed in tryptone saline media at pHs 5.5 and 7.5, since this is the pH range within which intraphagosomal bacteria would be subjected to the lethal action of 37K CAP (7). We found that when tested at 5 μg of 37K CAP per ml, several gram-negative bacteria, but none of the gram-positive bacteria, were sensitive to 37K CAP (Table 1). The susceptible gram-negative bacteria included *A. lwoffii*, *E. coli*, *N. gonorrhoeae*, *P. aeruginosa*, *P. cepacia*, *S. typhimurium*, *S. typhi*, and *S. sonnei*. These gram-negative bacteria were found to be less resistant to 37K CAP at pH 5.5, than at pH 7.5. Three gram-negative bacteria (*P. mirabilis*, *P. vulgaris*, and *S. marcescens*) were as resistant as the gram-positive bacteria to 37K CAP at either pH.

LPS-dependent resistance of *S. typhimurium* to 37K CAP. We have demonstrated recently the ability of 37K CAP to bind to the inner core or lipid A region of bacterial LPS (Wollenweber et al., submitted). We have suggested that the interaction of 37K CAP with this region of LPS is necessary for the initiation of bactericidal activity. The ability of 37K CAP to interact with the lipid A or inner core region of LPS enables us to predict that mutations decreasing the length of the core oligosaccharide would expose 37K CAP receptors on the bacterial surface, thus rendering deep-rough LPS mutants less resistant to 37K CAP than strains bearing more complete LPS. To test this prediction directly, we examined the 37K CAP resistance of a panel of nearly isogenic *S. typhimurium* strains which either synthesize core oligosaccharides containing various amounts of carbohydrate or possess an O antigen. We examined the 37K CAP resistance of such strains in tryptone saline media at pH 5.5 or 7.5. This was done because earlier studies by Rest et al. (6) showed that fully smooth strains, such as LT2, were less resistant to crude granule extracts in relatively acidic media, whereas deep-rough LPS mutants (Re and Rd_1 LPS chemotypes) were equally sensitive to crude extracts in acidic (pH 5) or basic (pH 7.5) media. We found that in pH 5.5 tryptone

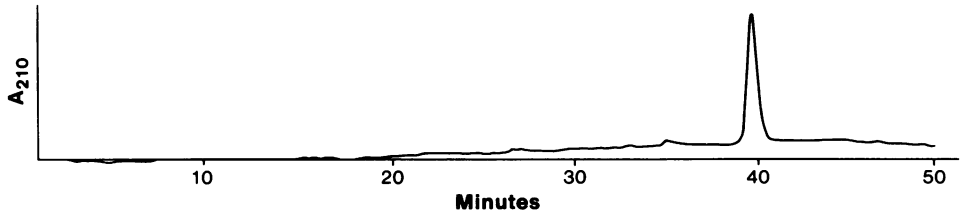

FIG. 2. RP-HPLC analysis of 37K CAP. Five micrograms of 37K CAP (in 0.2 M sodium acetate buffer) was applied to a Waters C-18 analytical RP-HPLC column (no. 096739). A linear gradient of 0.1% (vol/vol) trifluoracetic acid (buffer A) and 60% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid (buffer B) was applied to the column at a flow rate of 1 ml/min. The A_{210} was determined and showed a single peak emerging from the column at approximately min 39.

saline all of the strains were sensitive to 37K CAP; the level of resistance was smooth > Ra > Rd_1 > Re (Fig. 5). We were interested to find that all of the strains examined (with the exception of the Re mutant strain SH 7518) were substantially more resistant to 37K CAP when incubated in pH 7.5 tryptone saline media as opposed to pH 5.5 media. In the experiment described above, we also compared the resistance of isogenic strains bearing an Re LPS chemotype but differing in the *pmrA* locus. This locus is known to increase resistance to polymyxin B, probably by decreasing the electronegativity of the lipid A or inner core region of LPS (13). The parental strain bearing the wild-type $pmrA^+$ allele was readily killed by 37K CAP, regardless of pH, while strain SH 7519 bearing the mutant *pmrA* allele was substantially more resistant to 37K CAP at pH 7.5 than at pH 5.5. In fact, the level of resistance of strain SH 7519 to 37K CAP at pH 7.5 resembled that of fully smooth strain LT2.

DISCUSSION

In this communication we describe the antimicrobial potency of the arginine-rich (8) protein 37K CAP prepared from extracts of human PMN granules. When purified to homogeneity by silver-stained SDS-PAGE and RP-HPLC (Fig. 1 and 2), 37K CAP had potent in vitro antimicrobial activity against a wide spectrum of gram-negative bacteria. However, at the concentration evaluated, 37K CAP was found to be ineffectual against gram-positive bacteria (Table 1). Most important, we found that mildly acidic conditions believed similar to the environment of the mature PMN phagolysosome favored the antimicrobial capacity of 37K CAP.

The finding that 37K CAP-sensitive gram-negative bacteria were rendered uniformly more susceptible to the lethal action of 37K CAP at pH 5.5 than at pH 7.5 indicates that the developing phagolysosome is a suitable environment for the bactericidal activity of 37K CAP. In this regard, studies by Segal et al. (7) have shown that although there is an initial rise in the pH (ca. pH 7.2) of the phagolysosome shortly after phagocytosis, the environment rapidly becomes acidified to ca. pH 5.5 to 6.0. Accordingly, 37K CAP is a candidate for intraphagosomal killing of bacteria during not only the early stages after phagosome-lysosome fusion but also subsequent periods when the pH of the maturing phagolysosome approaches the lower estimates of 5.5 to 6.0 (7, 10).

The ability of 37K CAP to bind specifically and with high affinity to the lipid A or inner core region of LPS (Wollenweber et al., submitted) is consistent with our observation that only gram-negative bacteria are susceptible to its lethal action. Binding to this region of LPS is due likely to the availability of electronegative charges provided by the 4′ phosphate of lipid A or phosphates in the inner core region (8). Conversely, binding could be antagonized by positively charged groups within the lipid A or inner core region of LPS. Indeed, we have shown (8) that increased substitution (due to expression of *pmrA* [13]) of the 4′ phosphate of lipid A by 4-amino-1-deoxyarabinose and increases in the concentration of phosphorylethanolamines in the inner core region correlate with increased resistance *S. typhimurium* to 37K CAP. Binding of 37K CAP to LPS via ionic interactions could also be modulated by pH. Thus, at lower pH values, the total positive charge of 37K CAP would be increased, thereby promoting greater ionic interactions. This interaction may explain, in part, why 37K CAP exerts greater bactericidal activity at pH 5.5 rather than pH 7.5. Alternatively, more acidic conditions could damage bacterial outer membranes so as to facilitate binding of 37K CAP.

Our model of 37K CAP interaction with LPS suggests two predictions. First, deep-rough LPS mutants should be more sensitive than less rough or smooth strains to 37K CAP, owing to increased exposure of negatively charged groups in the lipid A or inner core region. Second, increases in the positive charge of this region of LPS due to *pmrA* (or other mutations) should increase bacterial resistance to 37K CAP. In support of the first prediction, we found that nearly isogenic strains of *S. typhimurium* LT2 differing in LPS composition became progressively less resistant to 37K CAP as the length of the core oligosaccharide decreased to the Re LPS chemotype (Fig. 5). In support of the second prediction,

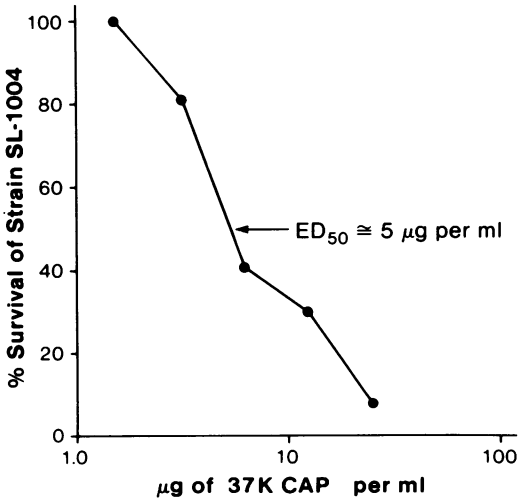

FIG. 3. Killing of *S. typhimurium* SL-1004 by 37K CAP. Using the standard bactericidal assay described in the text, we found that approximately 5 μg of 37K CAP per ml was required to achieve a 50% effective dose (ED_{50}). Each datum point represents the average value of three determinations.

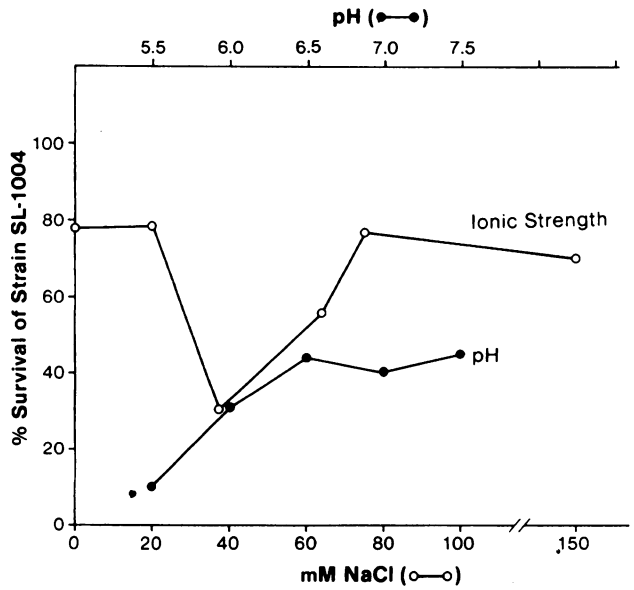

FIG. 4. The bactericidal capacity of 37K CAP is influenced by the pH and ionic strength of the incubation medium. *S. typhimurium* SL-1004 was exposed to 5 μg of 37K CAP per ml in tryptone saline altered in pH (●) or ionic strength (○). The optimal pH was ca. 5.5, whereas the optimal ionic strength was ca. 37.5 mM NaCl.

we showed earlier (8) that the Rb_1 *pmrA* mutant (SH 7426) of strain SH 9178 (Rb_1 LPS chemotype but *pmrA*$^+$) was substantially more 37K CAP resistant than was strain SH 9178. In the present study, we found that Re mutants differing at *pmrA*$^+$ differed considerably in 37K CAP resistance at pH 7.5 but not pH 5.5. Since these strains are isogenic and are thought to differ only in the chemical composition of the lipid A or inner core region (13), it is likely that acidic conditions favor the binding of 37K CAP to lipid A or the inner core region. Since the electronegativity of this region is decreased in the *pmrA* strain (13), acidic conditions, such as pH 5.5, rendering 37K CAP more electropositive, would presumably enhance ionic interactions of 37K CAP with the available negatively charged groups in the lipid A or inner core region (13). Expression of *pmrA*$^+$ in the Re mutant, on the other hand, would result in the availability of greater numbers of electronegative groups, perhaps permitting efficient binding of bactericidal quantities of 37K CAP regardless of pH. This hypothesis seems to be the simplest explanation for our results. We are currently investigating the capacity of this and other CAPs to bind to microbial cell surfaces under a variety of environmental conditions, including pH.

The presumed interaction of 37K CAP with the lipid A or inner core region of LPS in general and with the 4′ phosphate of lipid A via ionic interactions in particular might in part explain why *P. mirabilis* is completely resistant to 37K CAP. The lipid A 4′ phosphate of this bacterium is completely substituted by 4-amino-1-deoxyarabinose (9). Whether the 37K CAP resistance of *P. mirabilis* and other gram-negative bacteria, e.g., *S. marcescens*, is due solely to the inability to bind 37K CAP is not yet known.

The number of granule proteins now reported to exert in vitro antimicrobial activity includes, in addition to 37K CAP, 57K CAP (8), the bactericidal permeability-increasing protein (BPI) prepared by Weiss et al. (14), the chymotrypsin-like serine protease cathepsin G (5), and three lower-molecular-weight peptides described as "defensins" (3). It is interesting that the defensins (3), cathepsin G (5), and BPI (14) are reported to be optimally active at neutrality, whereas 37K CAP functions best at acidic pH values. Thus, 37K CAP may be responsible for a significant degree of nonoxidative killing of bacteria as the pH of the maturing phagolysosome decreases. Results from this and other studies, therefore, suggest that the presence of several CAPs with different pH optima would permit human PMNs to kill bacteria by nonoxidative means for an extended period following engulfment of bacteria.

TABLE 1. Selective bactericidal activity of 37K CAP against certain gram-negative bacteria

Resistance or sensitivity of gram-negative bacteria	% Survival[a] at pH:	
	5.5	7.5
Sensitive		
Acinetobacter lwoffii	34	80
Escherichia coli	4	60
Neisseria gonorrhoeae	38	53
Pseudomonas aeruginosa	36	100
Pseudomonas cepacia	39	72
Salmonella typhi	14	40
Salmonella typhimurium (LT2)	42	93
Shigella sonnei	29	82
Resistant		
Proteus mirabilis	100	95
Proteus vulgaris	100	99
Serratia marcescens	100	100
Bacillus subtilis	100	100
Staphylococcus aureus (Wood)	100	100
Staphylococcus epidermidis	100	92
Streptococcus pneumoniae	98	98
Streptococcus pyogenes	100	98

[a] Bactericidal assays used 5 μg of 37K CAP per ml. Assays were performed in pH 5.5 and 7.5 tryptone saline media. All values represent average results from at least three separate determinations.

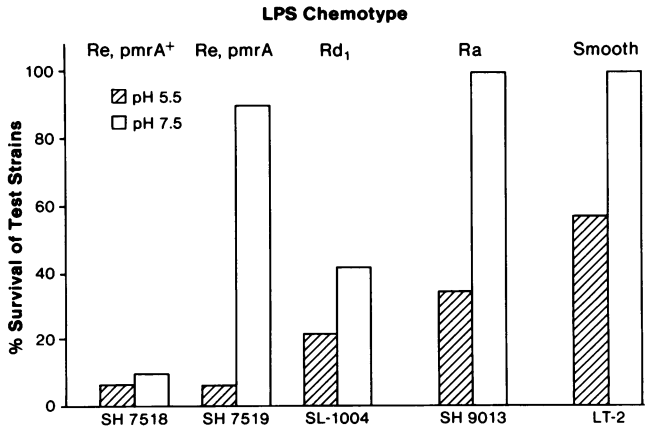

FIG. 5. *S. typhimurium* exhibits LPS-dependent resistance to 37K CAP at a pH range resembling that of the maturing phagolysosome. Nearly isogenic mutants of strain LT2 bearing Re, Rd, or Ra LPS chemotypes, together with strain LT2, were exposed to 5 μg of 37K CAP per ml in tryptone saline media (pHs 5.5 and 7.5). Note that, with the exception of the Re strain SH 7518, all of the test strains were less 37K CAP resistant at pH 5.5 than at pH 7.5. The Re strain bearing the *pmrA* allele (strain SH 7519) was readily killed by 37K CAP at pH 5.5 but greatly resisted its action at pH 7.5.

ACKNOWLEDGMENTS

We thank V. C. Onunka for excellent technical assistance and Joy Royer for skillful preparation of this manuscript. We also thank R. Vogler for his assistance in procuring human PMN granulocytes.

This work was supported by Public Health Service grants AI 17662 (to J.K.S.) and AI 21150 (to W.M.S.) from the National Institute of Allergy and Infectious Diseases.

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