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Immunology

Pulse labeling of small nuclear ribonucleoproteins *in vivo* reveals distinct patterns of antigen recognition by human autoimmune antibodies

(systemic lupus erythematosus/mixed connective tissue disease/Sm and RNP sera)

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ABSTRACT **Antibodies directed against small nuclear ribonucleoprotein (snRNP) particles are found in the Sm and RNP autoimmune sera from numerous patients with systemic lupus erythematosus (SLE) and mixed connective tissue disease (MCTD). These two reactivities differ in disease distribution as well as antigen specificity. Although sera from both of these autoimmune syndromes contain snRNP reactive antibodies, distinctions in antigen binding specificity have been difficult to define because of the particulate nature of the snRNP antigen. To overcome this problem, while retaining the antigen in a native state, cells were pulse-labeled with [^{35}S]methionine for 8 min to generate radioactive snRNP proteins in forms reflecting incomplete *de novo* particle assembly. Immunoprecipitation of snRNP antigen prepared in this manner revealed clearly distinct patterns of Sm and RNP immunorecognition. While Sm sera precipitated all eight labeled snRNP proteins, RNP antibodies precipitated only two of the eight. However, a brief pulse followed by periods of cold chase demonstrated that RNP sera can eventually coprecipitate all components of the complete particle. In addition to antibodies to the other six snRNP peptides, all Sm sera tested have been found to contain the RNP-like reactivity with snRNP proteins A and C. RNP reactivity with these two components is of particular interest because these proteins are unique in the metabolism of snRNPs. Defining and distinguishing the precise peptides recognized by Sm and RNP antibodies has helped to clarify the biochemical basis of the standard laboratory tests for these antigen reactivities.**

Since their description in 1966 (1), antigens from nuclear extracts reactive with autoimmune sera have become the subject of considerable investigation. The observation that these antigens are ribonucleoprotein particles (2) containing U series small nuclear RNAs (snRNPs) (3) accompanied the proposals (4, 5) that these snRNPs may participate in the splicing of messenger RNA precursors. Parallel to their importance in molecular biology, snRNPs have been intensely studied by numerous laboratories interested in their naturally occurring antigenicity in human diseases (2, 3, 6–10). This curiosity stems from the identification of several antibody reactivities (11) found frequently in sera of systemic lupus erythematosus (SLE), mixed connective tissue disease (MCTD) (9), and other conditions (12), which may be of some diagnostic and prognostic importance.

The two serotypes that display reactivity with snRNPs are Sm and RNP. They have been distinguished by virtue of an apparent RNase sensitivity of the antigen recognized by RNP sera (2, 9, 13). Analysis of the protein constituents of snRNPs has resulted in differing, although likely compatible, reports on the precise components of these particles. *In vitro* translation and assembly of snRNP proteins have been described (14, 15) as well as fractionation of the RNAs that encode the snRNP proteins (14). These studies together with the reports (16, 17) of nonimmunological snRNP particle purification have allowed at least eight separately translated proteins to be identified as snRNP constituents.

Identification of which species are specifically recognized by Sm and RNP serum antibodies has been plagued by difficulties related to the noncovalent interactions that hold snRNP particles together. Therefore, immunoprecipitation of endogenously labeled snRNPs has been of limited value in delineating distinctions between peptide recognition by Sm and RNP sera. Studies from this laboratory (6) using detergent treatment and immunoblot analyses revealed several differences in the serologies. However, identification of all antigenic peptides requires native undenatured proteins. In this study, such snRNP peptides were generated by brief [^{35}S]methionine pulse labeling of human cells in culture. By using antigen prepared in this manner, it was shown that Sm sera react with all snRNP protein components, whereas RNP sera recognize only two of these components.

MATERIALS AND METHODS

Patients and Sera. Sm and RNP serological determinations were carried out by the passive hemagglutination assay described by Tan and Peebles (18). Reference Sm and RNP sera were obtained from E. Tan (Scripps Clinic and Research Institute, La Jolla, CA) and V. Agnello (New England Medical Center, Boston, MA).

Cells and Cell Growth. The human myeloid line K562 was grown in RPMI 1640 medium (Microbiological Associates, Walkersville, MD) supplemented with 10% fetal bovine serum (GIBCO), 2 mM glutamine, and antibiotics (complete medium).

***In Vivo* Labeling.** Extended labeling was carried out by resuspending washed cells at 10^6 per ml in methionine-free RPMI 1640 medium supplemented with 10% fetal bovine serum, glutamine, and antibiotics as above (methionine-free medium). To this, 0.5 μg of methionine per ml was added together with 100 μCi of [^{35}S]methionine (800–1000 Ci/mM, New England Nuclear; 1 Ci = 37 GBq) per ml. After incubation for 15 hr, cells were washed three times in ice-cold Dulbecco's phosphate-buffered saline (P_i/NaCl) and lysed. Pulse and pulse–chase procedures were carried out on cells starved for 2 hr in methionine-free medium. Methionine-starved cells (usually 5×10^7 cells) were pelleted, and 100 μl of methionine-free medium supplemented with 500 μCi of [^{35}S]methionine was added. The cell suspension was incubated for eight min at 37°C, followed by immediate resuspension in 50 ml of ice-cold P_i/NaCl. In chase experiments, pulsed cells were resuspended in complete medium at a den-

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Abbreviations: SLE, systemic lupus erythematosus; MCTD, mixed connective tissue disease; snRNP, small nuclear ribonucleoprotein.

sity of 10^6 cells per ml. During chase, cells were incubated at 37°C, and appropriate aliquots were removed, washed in ice-cold P_i/NaCl, and extracted at the time points listed in the legend to Fig. 4.

Cell Lysis and Immunoprecipitation. After three washes in ice-cold P_i/NaCl, cells were swelled for 10 min on ice in 10 mM KCl/10 mM Tris chloride, pH 7.6/5.0 mM iodoacetamide/0.5 mM phenylmethylsulfonyl fluoride/1.0 mM EDTA. Cells were then lysed by 12 strokes with a Dounce homogenizer. NaCl was added to 0.2 M, and the lysate was clarified by centrifugation at 12,000 × *g* for 5 min. Immunoprecipitation of the lysate was carried out by the addition of 5–10 μl of appropriate patient serum per immunoprecipitate. Cells were lysed at 10^6–10^7 cells per ml, and each immunoprecipitate was derived from 1–5 × 10^6 cells. After 1 hr at 4°C, 40 μl of protein A-Sepharose 4B (Pharmacia) was added, and the tubes were rotated for an additional hour at 4°C. Immunoprecipitates were washed four times with 0.5% NaDodSO$_4$/2.5% Triton X-100/50 mM Tris chloride, pH 7.5/0.15 M NaCl/1.0 mM EDTA/0.25 M sucrose, followed by two washes with 50 mM Tris chloride, pH 7.5/0.15 M NaCl/1.0 mM EDTA/1% Trasylol.

***In Vitro* Translation of snRNP Proteins.** RNA extraction, poly(A) selection, and *in vitro* translation were carried out as described (14, 19) with wheat germ extract. Immunoprecipitation was carried out by adjusting translation products to 1% Triton X-100/0.1 M NaCl/25 mM Tris chloride, pH 7.4/5.0 mM EDTA/2.5% Trasylol containing 2.5 mg of ovalbumin 0.1 μg each of antipain, leupeptin, and pepstatin per ml and adding 5–10 μl of Sm or RNP serum.

Gel Analysis. All washed protein A-Sepharose immunoprecipitates were eluted in 0.25 M sucrose/2.0% NaDodSO$_4$/0.1 M Tris chloride, pH 7.4/5.0 mM EDTA/0.1 M dithiothreitol/0.005% bromphenol blue. After 5 min at 100°C, eluates were removed and applied to 15% NaDodSO$_4$-polyacrylamide gels (20) and run at 25 mA for 14 hr. Gels were stained in Coomassie blue, destained, treated with diphenyloxazole for fluorography (21), and dried prior to autoradiography.

RESULTS

Sm and RNP Antigen Recognition. Extended *in vivo* labeling of snRNPs was carried out by incubation of K562 cells for 15 hr in the presence of [^{35}S]methionine. Lysis and immunoprecipitation resulted in the identification of eight previously described proteins uniquely precipitated by both Sm and RNP sera but not by normal human serum (Fig. 1). Although various higher molecular weight bands have been seen as well, including several uniquely recognized by certain sera, it has been difficult to correlate their presence with either Sm or RNP serology (6). The eight precipitated proteins range in molecular weight from 32,000 to 9,000 daltons and are labeled A–G. Although both Sm and RNP sera precipitated all eight proteins, reproducible differences in relative intensities of certain bands were seen. Sm sera precipitated an additional quantity of B, B′, and D proteins, whereas RNP sera precipitated additional C protein.

Previous work has indicated that bands A–G exist in complex particles (14–17) that could be dissociated by mild detergent treatment during extraction of antigen from labeled cells (6). Furthermore, unique and distinguishable subsets of these eight proteins (from dissociated particles) are recognized by Sm and RNP sera, although detergent denaturation destroys reactivity with several of the proteins. As an alternative to particle disruption by detergents, incompletely assembled snRNPs might facilitate the analysis of Sm and RNP serological reactivities with native snRNP components. Such reactivities would be identified by immunoprecipitation of extracts from cells pulsed briefly with [^{35}S]methionine rather than labeled for an extended period of time. The results of immunoprecipitation after an 8-min pulse of [^{35}S]methionine are shown in Fig. 2. The three Sm sera shown precipitated all eight snRNP proteins, whereas the

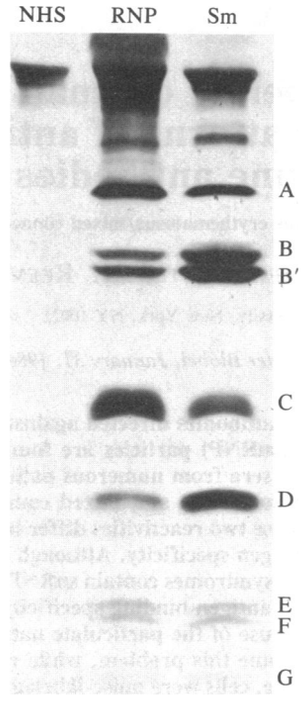

FIG. 1. Immunoprecipitation of snRNP proteins labeled for an extended period. K562 cells were labeled with [^{35}S]methionine for 15 hr and lysed; the lysate was precipitated with normal human serum (lane NHS), Sm, and RNP sera. SnRNP proteins were resolved on a 15% polyacrylamide slab gel. The snRNP proteins A–G are identified.

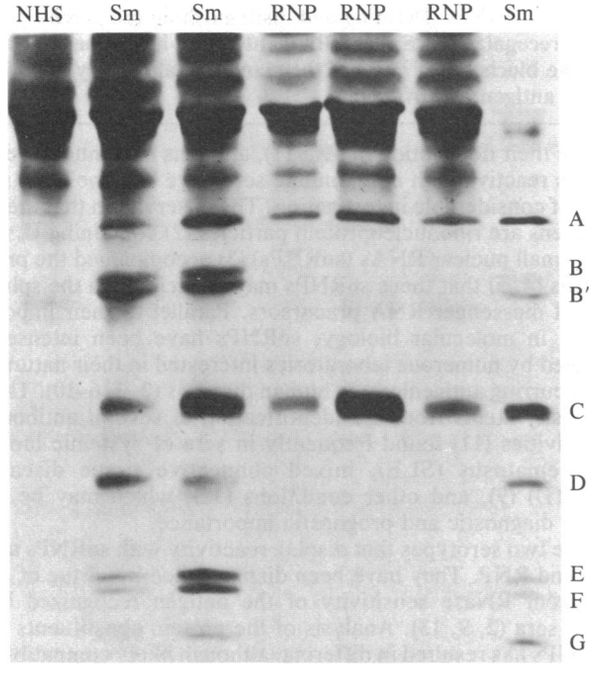

FIG. 2. Immunoprecipitation of pulse-labeled snRNP proteins. K562 cells were starved of methionine for 2 hr and then pulsed for 8 min with [^{35}S]methionine. After cell lysis, the snRNP proteins were immunoprecipitated with normal human serum (lane NHS), three different Sm sera, and three different RNP sera.

three RNP sera precipitated proteins A and C only. Normal human serum (Fig. 2, lane NHS) precipitated none of these proteins. Thus, although all eight particle proteins incorporate [^{35}S]methionine during an 8-min pulse (as illustrated by their precipitation with the Sm sera), only a subset of these proteins, bands A and C, are precipitable by RNP sera. Pulses as short as 2 min gave identical results (data not shown). Studies of over 20 Sm and RNP sera, including standards from other institutions (kindly provided by E. Tan and V. Agnello) demonstrated the same distinct Sm and RNP patterns of reactivity in all cases.

An additional source of native snRNP antigen, most of which is in a nonassembled state, is available in products of *in vitro* translation of poly(A)$^+$ RNA. Wheat germ extract (19) was utilized for *in vitro* translation of K562 poly(A)$^+$ RNA, followed by immunoprecipitation with Sm and RNP sera. Fig. 3 shows typical results of such experiments. Sm serum precipitated all eight proteins, whereas RNP serum precipitated only proteins A and C. Analysis of *in vivo* pulsed and *in vitro* synthesized snRNP proteins thus demonstrates the same distinguishable patterns of Sm and RNP antigen recognition.

Pulse–Chase Analysis of snRNP Antigenicity. Since RNP antibodies precipitated only a subset of the snRNP proteins after an *in vivo* 8-min pulse, it seemed likely that, during this time period, snRNP assembly using newly translated (and labeled) proteins had not been completed. That is, either the labeled proteins were precipitating as entirely separate entities or they were precipitating by virtue of intermediate associations between themselves and/or other unlabeled snRNP proteins. If either of these possibilities were true, it should be possible to "chase" the labeled proteins into a state that reflects complete *de novo* particle assembly. This was tested by pulsing cells for 8 min, followed by periods of cold chase. Comparison of proteins precipitated by RNP serum after several periods of chase are seen in Fig. 4. The time point representing no chase (0 hr) shows the same distinct pattern of RNP immunoprecipitation as seen in Figs. 2 and 3. Sm serum precipitates proteins A–G, whereas RNP serum precipitates A and C only. However, increasing periods of cold chase, were accompanied by the eventual appearance of all eight bands in the RNP immunoprecipitates. Bands B and B′ appear earliest, followed by bands D, E, F, and G, all of which are present in the RNP-chase by 10 hr.

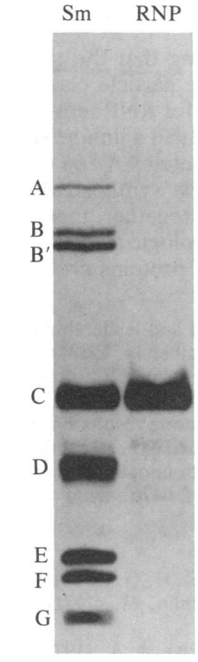

FIG. 3. Sm and RNP recognition of snRNP proteins generated by *in vitro* translation. Poly(A)$^+$ RNA from K562 cells was translated *in vitro* with wheat germ extract. Products were immunoprecipitated with Sm (left lane) and RNP (right lane) sera.

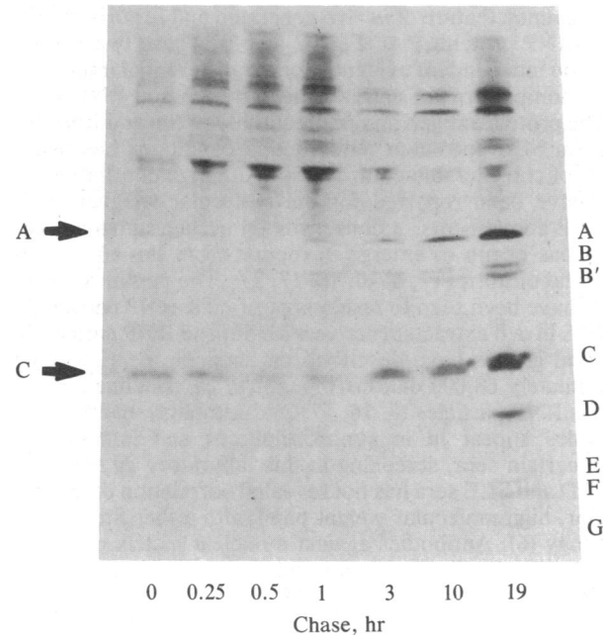

FIG. 4. Pulse–chase analysis of snRNP recognition by RNP sera. After 2 hr of methionine starvation and an 8-min [^{35}S]methionine pulse label, K562 cells were resuspended in medium containing unlabeled methionine and incubated further at 37°C. At 0, 0.25, 0.5, 1, 3, 10, and 19 hr, aliquots of cells were removed and lysed, and their snRNP proteins were immunoprecipitated with RNP serum.

DISCUSSION

The previous inability to distinguish the fine nature of antigen recognition of Sm sera from RNP sera is in large part due to the fact that snRNP antigens are complex particles. Antibody recognition of any component in the particle results in immunoprecipitation of the entire particle. Thus, when the antigen is radioactively labeled by prolonged incubation with [^{35}S]methionine, little if any difference has been seen in the patterns obtained with Sm and RNP sera (Fig. 1). Previous attempts to disrupt the particles using 0.5% $NaDodSO_4$ treatment have revealed differential recognition of several snRNP peptides by Sm and RNP sera (6). Likewise, when using $NaDodSO_4$ gels and immunoblots, several of the snRNP peptides have been observed to be uniquely recognized by Sm and/or RNP sera and monoclonal antibodies (6, 8, 22). However, several of the peptides were not recognized, and the possibility remained that all snRNP peptides are antigenic but that $NaDodSO_4$ treatment seriously alters antigenicity.

For these reasons, the present study was initiated to analyze *in vivo* synthesized, native snRNP peptides. To circumvent the need for particle disruption or detergent treatment, the strategy involved brief pulse-labeling with [^{35}S]methionine. By this method of analysis, a clear distinction is seen between Sm and RNP serological immunorecognition (Fig. 2). Whereas Sm sera precipitate all snRNP components, only two of these peptides, A and C, appear in RNP immunoprecipitates. Evidence that the distinction depends on lack of complete *de novo* particle assembly is seen in Fig. 4. With cold chase, all snRNP peptides eventually appear in the RNP immunoprecipitates.

The selective recognition of proteins A and C by RNP sera

is a common feature of *in vivo* generated and *in vitro* translated snRNP proteins (14) (Figs. 2 and 3). These two systems provide independent evidence for the differential immunorecognition patterns of native antigen by Sm and RNP sera.

The protein antigen immunoreactivity of Sm and RNP sera seen in SLE and other autoimmune conditions has been a topic of considerable controversy. Although conflicting values have been reported for the molecular weights of the snRNP components, a consensus on certain antigenic peptides has begun to emerge in reports from this (6, 14) and other laboratories (7, 8, 10, 16, 17, 23). The peptides labeled A–G have been seen to reside in purified snRNP particles as well as in cell extracts reactive with Sm and RNP antibodies. Several groups have described an antigenic peptide of approximately 68,000 daltons thought to be contained in certain snRNP particles (8, 16, 17, 23). Although several larger peptides appear in immunoprecipitates and immunoblots with certain sera, screening in this laboratory of numerous MCTD and SLE sera has not revealed correlation of any particular, high molecular weight band with either Sm or RNP serology (6). Antibodies against a nuclear matrix protein of approximately the same molecular weight have been described in many MCTD sera (24). Further studies regarding the identity and immunogenicity of these larger peptides are currently in progress, utilizing several recently generated monoclonal antibodies (unpublished results). Experiments in this laboratory have not demonstrated the presence of any additional snRNP peptides in the range of 15,000–33,000 daltons (other than A–G) as reported by several other groups (16, 17). Although occasional immunoprecipitates have revealed such additional bands, comparison of total lysate proteins generally has revealed those species to correspond to very abundant cellular proteins. Lack of these bands in immunoprecipitates of *in vitro* translation products from size-fractionated poly(A)$^+$ RNA (14) supports this notion. However, a notable exception is the methionine-deficient protein discovered by Kinlaw *et al.* (25), which was not examined in this study.

An understanding of the peptide antigen recognition by Sm and RNP sera is of considerable value in light of the common clinically used assays for these sera. Whereas RNP reactivity has traditionally been distinguished from Sm by RNase sensitivity in hemagglutination and immunodiffusion assays (13, 26), it is of interest that immunoprecipitation of RNase-treated snRNPs produces an identical change in antigen recognition by the two sera. Loss of the protein A immunoprecipitation occurs for both Sm and RNP sera (14). U1 small nuclear RNA has been shown by Wieben *et al.* (15) to be critical for protein A antigenicity. However, by using very sensitive immunoblots, a small amount of protein A reactivity—presumably in the absence of RNA—has been observed by Petterson *et al.* (22). Thus, the finding that RNP sera recognize only proteins A and C may help to explain the biochemical basis for the hemagglutination assay, since essentially only protein C reactivity remains for RNP sera in RNase-treated snRNPs. For reasons of valency, three-dimensional configuration, or chemical treatments of antigen, protein C may be poorly reactive in snRNP particles in agar diffusion plates (2) or when attached chemically to erythrocytes (18)—particularly since the most common source of antigen is an acetone extract of rabbit thymus. Antibody to protein C may poorly hemagglutinate or precipitate with antigen under these conditions. If protein C recognition is not predominant in these assays, the protein A antigenicity would be the critical antigen for RNP antibody-induced hemagglutination or agar precipitin formation. RNase treatment of snRNPs, then, would be expected to abolish RNP reactivity. However, additional recognition by Sm serum of proteins B, B′, D, E, F, and G would still allow for Sm reactivity in these assays, regardless of RNase. This interpretation is further supported by the observation that, with an ELISA assay, RNP recognition may be somewhat diminished (but is by no means abolished) by pretreatment of snRNPs with RNase (unpublished results). White and Hoch (8) also have demonstrated RNP antigen recognition in the absence of associated RNA from rabbit thymus acetone extract.

Several groups (16, 17) have reported the isolation of two classes of snRNP particles, one of which lacks proteins A and C. This class contains the U2–6 small nuclear RNA species, whereas the particles containing proteins A and C contain U1 RNA. Aside from a 68,000-dalton peptide, the other six proteins (B, B′, D, E, F, and G) are common to both classes of snRNPs.

In the light of the finding that RNP sera recognize proteins A and C only, whereas Sm sera recognize all eight components, early reports of the relationship between Sm and RNP antigens are better understood. Several groups (2, 9, 13) described RNP (also called "Mo") as an RNA-containing antigen. However, fractionation experiments failed to separate it from Sm-reactive material. This was in contrast to the ability to fractionate pure Sm-reactive material away from RNP/Sm-reactive antigen. Thus, pure Sm-reactive antigen likely corresponds to U2–6-containing snRNPs, which contain six peptides but lack proteins A and C. Sm/RNP-reactive antigen corresponds to U1-containing snRNPs, which react with RNP antibodies because of the unique presence in these particles of protein A (with its associated RNA) and protein C. An additional noteworthy observation concerns the finding that all Sm sera studied contain RNP-like reactivity, an observation that also has been made by use of immunoblots by Petterson *et al.* (22) and an ELISA assay (unpublished data). This clear presence of RNP-like antibodies in Sm sera raises questions regarding similarities and differences in the respective disease states. Although the determinants on proteins A and C recognized by the Sm and RNP antibodies could conceivably differ, it is striking that both sera lose reactivity with protein A after RNase treatment of antigen (14). Thus, at least one characteristic of the recognition is common to both sera. Relevant to this question is the recent observation of a temporal decrease in only the non-A and -C Sm-specific antibodies in a SLE patient (unpublished data).

It is highly interesting that the two peptides (A and C) which distinguish these particle classes contain the precise antigenic determinants for RNP sera. Analysis of *in vivo* particle assembly has revealed a unique sequence of events that further distinguishes proteins A and C in the kinetics of their incorporation into newly synthesized snRNP particles (unpublished data). Taken together, these considerations would suggest that the immunologic as well as parallel biochemical uniqueness of A and C proteins are not coincidental.

The inspiration, insight, and leadership of Dr. Henry G. Kunkel (September 9, 1916–December 14, 1983) are affectionately recalled. The authors thank Drs. Nicholas Chiorazzi for useful discussions and Robert Lahita for Sm and RNP sera. Dr. Joan A. Steitz is gratefully acknowledged for sharing results prior to publication. Ms. Rosanne Wisniewolski provided expert technical assistance. W.H.R. is a Fellow of the Arthritis Foundation. This work was supported by Grants AI 10811 and AM 04761 from the National Institutes of Health.

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