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Immunology

Cellular requirements for tumor-specific immunity elicited by heat shock proteins: Tumor rejection antigen gp96 primes CD8+ T cells *in vivo*

(stress protein/effector phase/priming/macrophages/CD4+ T cells)

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ABSTRACT **Purified preparations of 96-kDa heat shock proteins (gp96) have been previously shown to elicit tumor-specific immunity to the tumor from which gp96 is obtained but not to antigenically distinct chemically induced tumors. The cellular requirements of gp96-elicited immunity have been examined. It is observed that depletion of CD8+, but not CD4+, T cells in the priming phase abrogates the immunity elicited by gp96. The CD8+ T cells elicited by immunization with gp96 are active at least up to 5 weeks after immunization. Depletion of macrophages by treatment of mice with carrageenan during the priming phase also results in loss of gp96-elicited immunity. In the effector phase, all three compartments, CD4+ and CD8+ T cells and macrophages, are required. Immunity elicited by whole irradiated tumor cells shows a different profile of cellular requirements. In contrast to immunization with gp96, depletion of CD4+, but not CD8+, T cells during priming with whole tumor cells abrogates tumor immunity. Further, ablation of macrophage function during priming or effector phases has no effect on tumor immunity elicited by whole cells. Our results suggest the existence of a macrophage-dependent and a macrophage-independent pathway of tumor immunity. Our observations also show that in spite of exogenous administration, vaccination with gp96 preparations elicits a CD8+ T-cell response *in vivo*, and it is therefore a useful method of vaccination against cancer and infectious diseases.**

Chemically induced tumors of inbred mice elicit individually distinct immunity in syngeneic hosts (see ref. 1). Heat shock proteins of 96-kDa size (gp96) have been suggested to mediate this antigenicity in the case of a number of methylcholanthrene-induced sarcomas (2–4) and a UV-induced fibrosarcoma (S. Janetzki, N. E. Blachere, and P.K.S., unpublished results). Administration of a purified gp96 preparation renders mice resistant to the tumor from which gp96 is isolated but not to an antigenically distinct tumor (2). In light of a lack of differences in the gp96 cDNA sequence among different tumors or among tumors and normal tissues, we suggested that the tumor-specific antigenicity of gp96 may derive not from gp96 *per se* but from low molecular weight peptides associated with gp96 (5, 6). Such peptides were recently shown to be associated with gp96, and it was demonstrated that gp96 is an ATPase (7). The ATPase activity suggests a possible mechanism of transfer of antigenic peptides from gp96 to major histocompatibility class I in the antigen-presentation pathway or upon immunization of mice with gp96 preparations.

The immunological circuitry that leads to tumor resistance upon immunization of mice with gp96 has been examined in the present studies. Our results show that immunization with tumor-derived gp96 leads to priming of tumor-specific CD8+ lymphocytes *in vivo* and that this priming requires participation of phagocytic cells. In these two crucial respects, the tumor resistance elicited by immunization with gp96 follows a pathway distinct from that seen upon immunization of mice with intact tumor cells.

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MATERIALS AND METHODS

Mice and Tumors. BALB/cJ female mice (6–8 weeks old) were purchased from The Jackson Laboratory and were maintained in a virus-free animal facility in the Mount Sinai School of Medicine. Methylcholanthrene-induced fibrosarcoma Meth A (BALB/cJ origin) was maintained in ascites form in BALB/cJ mice by weekly passage of 2 million cells.

Antibodies. Rat anti-mouse CD4 monoclonal antibody GK1.5 and anti-mouse CD8 monoclonal antibody (anti-Lyt 2.2; ref. 8) were obtained in ascites form from E. Nakayama (Okayama University School of Medicine, Okayama, Japan). Fluorescein isothiocyanate-coupled second antibodies adsorbed with mouse immunoglobulins were obtained from Caltag (South San Francisco, CA). Anti-grp94 (gp96) rat monoclonal antibody (SPA-850, clone 9G10) was purchased from StressGen Biotechnologies (Sidney, Canada).

Purification of gp96. gp96 was purified from Meth A cells as described (2). Briefly, a 60-ml pellet of Meth A cells was lysed in 240 ml of hypotonic buffer (30 mM $NaHCO_3$/0.5 mM phenylmethylsulfonyl fluoride, pH 7.1) by Dounce homogenization, and a 100,000 × *g* supernatant was obtained. The supernatant was applied to a concanavalin A-Sepharose (Pharmacia) column, and glycoproteins were eluted by 10% α-methylmannoside. The eluate was resolved on a Mono Q (Pharmacia) FPLC system equilibrated with 5 mM phosphate buffer (pH 7.0) and eluted by a 400–600 mM NaCl gradient. Fractions (1 ml) were collected and analyzed by SDS/PAGE and immunoblotting with anti-gp96 antibody. The biochemical purity and serological identity of a typical gp96 preparation used in tumor rejection assays are shown in Fig. 1. The preparations were devoid of the 110-kDa molecule reported by DeLeo *et al.* (9).

Tumor Rejection Assay. Mice were injected subcutaneously with 20 million irradiated tumor cells or 6 μg of gp96 in phosphate-buffered saline (PBS) twice at weekly intervals. Mice were challenged with 50,000–100,000 live tumor cells (as indicated in each experiment) on either flank after shaving. Two tumor diameters of each mouse, at right angle to each other, were measured every 5–7 days, and their arithmetic mean was determined to arrive at the average tumor diameter.

Depletion of T Cells or Macrophages *in Vivo*. Ascites fluid containing anti-CD4 or -CD8 antibody (25 μl) was diluted 1:8 with PBS and injected into the retro-orbital sinuses of mice. Mice were sacrificed at the indicated intervals and tested for

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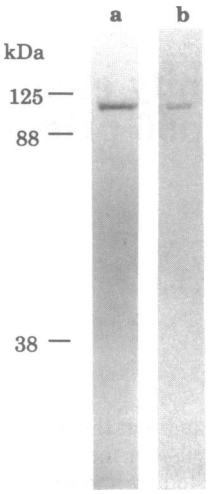

FIG. 1. Biochemically purified gp96 preparation (1 μg) derived from the Meth A sarcoma analyzed by SDS/PAGE followed by silver staining (lane a) or immunoblotting (lane b) as described in *Materials and Methods*.

depletion of the appropriate cell types by FACS analysis. Fig. 2 shows the kinetics of depletion and recovery of $CD4^+$ and $CD8^+$ T cells after treatment with the corresponding antibody. It is observed that administration of either antibody completely depletes splenic $CD4^+$ T cells or $CD8^+$ T cells almost immediately after injection of the antibody. The depletion remains complete for 2 weeks, after which the T-cell subsets gradually recover. The recovery of $CD4^+$ cells is more rapid than that of $CD8^+$ T cells such that at day 47, ≈50% of the original $CD4^+$ population and 27% of the original $CD8^+$ T cell population have recovered. Depletion and recovery of T cells in lymph nodes follow the same kinetics as seen in spleen (data not shown). We have used the information shown in Fig. 2 to plan the depletion experiments discussed in *Results* (see Fig. 3). For functional inhibition of macrophages, 1 mg of carrageenan (type II; Sigma) was injected i.p. (10).

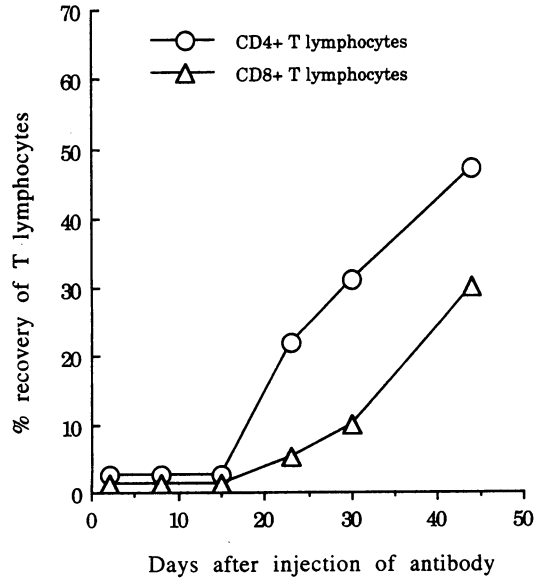

FIG. 2. Kinetics of depletion and recovery of $CD4^+$ and $CD8^+$ T lymphocytes in mice. The antibodies used and methods of depletion are described in *Materials and Methods*. Each point represents an average of two mice. The pattern shown is derived from the spleen; however, a similar pattern of depletion and recovery was observed in lymph node-derived T cells.

FACS Analysis. Spleen cells were washed twice with PBS containing 2% (vol/vol) fetal calf serum and suspended in the same medium. One million cells in 200 μl were incubated with fluorescein isothiocyanate-conjugated anti-CD8 antibody (Becton Dickinson; 1:200 dilution) for 30 min on ice and washed twice. For analysis of $CD4^+$ cells, cells were stained with GK1.5 antibody (1:200 dilution) followed by goat anti-rat IgG conjugated with phycoerythrin (Caltag, South San Francisco, CA; 1:4 dilution). Cells were washed, suspended in 1 ml of medium, and analyzed on a Profile fluorescence-activated cell sorter analyzer. Red blood cells were gated out.

RESULTS

The requirements for $CD4^+$ and $CD8^+$ T cells and for macrophages in tumor immunity elicited by intact Meth A cells or Meth A-derived gp96 were determined. As the requirements may differ in the priming and effector phases of the immune response, the two phases were examined independently. The strategy adopted for these experiments was to deplete $CD4^+$ or $CD8^+$ T cells or macrophages during a particular phase and monitor the effect of such depletion on tumor rejection in mice vaccinated with irradiated intact Meth A cells or Meth A-derived gp96.

Cellular Requirements in the Priming Phase. The tumor rejection assay used in our laboratory (2) involves two vaccinations (at day 0 and day 7) followed by a live tumor challenge 1 week after the last vaccination (day 14). As discussed in *Materials and Methods* and seen in Fig. 2, the anti-CD4 and anti-CD8 antibodies deplete the respective cell types within less than 2 days. Results shown by Debrick *et al.* (10) suggest that carrageenan also acts within a similar duration. Antibodies or carrageenan were injected immediately before vaccination (i.e., on day −1). The mice remain completely depleted of $CD4^+$ and $CD8^+$ T cells for 14 days after antibody administration and begin to repopulate very slowly. This leads to the problem that, if mice were to be challenged on day 14 (as in our standard tumor rejection assays) after being injected with depleting antibodies on day −1, the depleted state will not be restricted to the priming phase but will extend to at least a part of the effector phase as well. To distinguish between the two phases, tumor challenge should be given at a time when a sufficient proportion of $CD4^+$ and $CD8^+$ T cells have recovered. Our experiments in an allogeneic system suggest that repopulation of ≈25% of the original population is sufficient

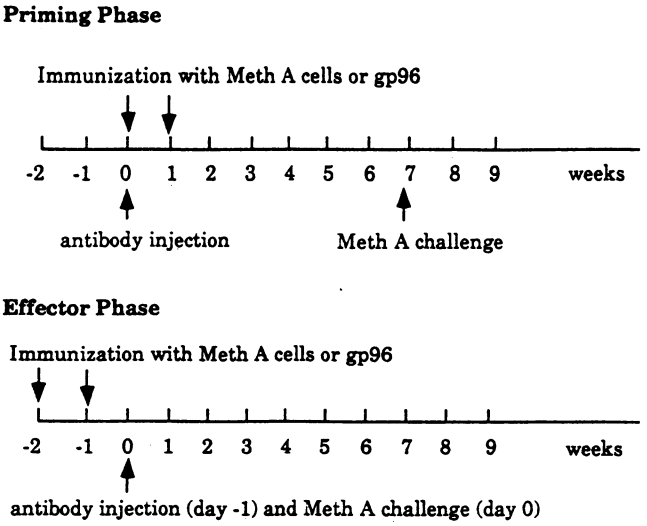

FIG. 3. Experimental protocol used for depletion of $CD4^+$ or $CD8^+$ T cells or of macrophages during the priming and effector phases of tumor immunity elicited by immunization with intact Meth A cells or Meth A-derived gp96. See text for details.

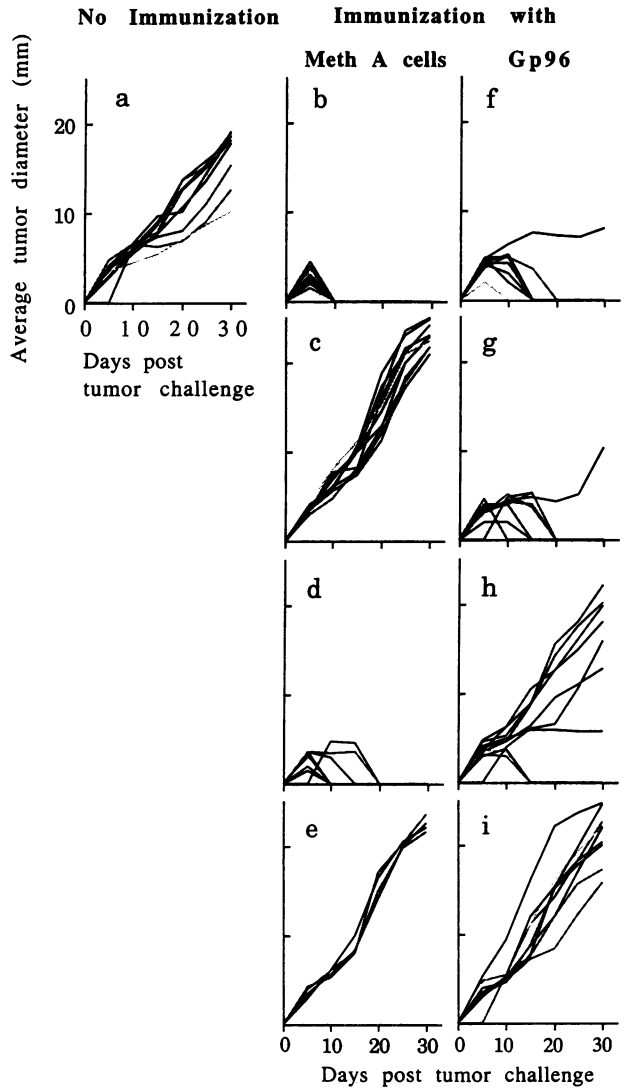

FIG. 4. Requirements of CD4+ and CD8+ T cells during the priming phase of tumor immunity generated by vaccination of mice with irradiated Meth A cells (*b*, *c*, *d*, and *e*) or with Meth A-derived gp96 (*f*, *g*, *h*, and *i*). Tumor growth in unimmunized mice is shown in *a*. Immunized mice were not depleted of either subset (*b* and *f*), CD4+ cells alone (*c* and *g*), CD8+ cells alone (*d* and *h*), or both CD4+ and CD8+ cells (*e* and *i*). All mice were challenged with 50,000 Meth A cells. The antibodies and methods used for depletion are described in *Materials and Methods*. The experimental protocol shown in Fig. 3 was followed. Each line represents the kinetics of tumor growth in a single mouse.

for a significant response (data not shown). Data shown in Fig. 2 indicate that 25–50% of T cells recover by ≈45 days after antibody injection. A significant proportion of functionally active macrophages are also expected to repopulate by 45 days or less (11). The protocol shown in Fig. 3 was therefore followed.

It is observed (Fig. 4) that mice immunized with Meth A-derived gp96 or with intact Meth A cells remain resistant to Meth A challenges 47 days after the last immunization. This shows that the immunity elicited by gp96 is indeed long-lasting. It is further observed that depletion of CD8+ T cells but not of CD4+ T cells leads to abrogation of tumor immunity elicited by gp96. In contrast, in the case of immunization with intact Meth A cells, depletion of CD4+ T cells but not of CD8+ T cells leads to abrogation of tumor immunity. Depletion of both CD4+ and CD8+ T cells leads to abrogation of tumor immunity elicited by intact cells or gp96.

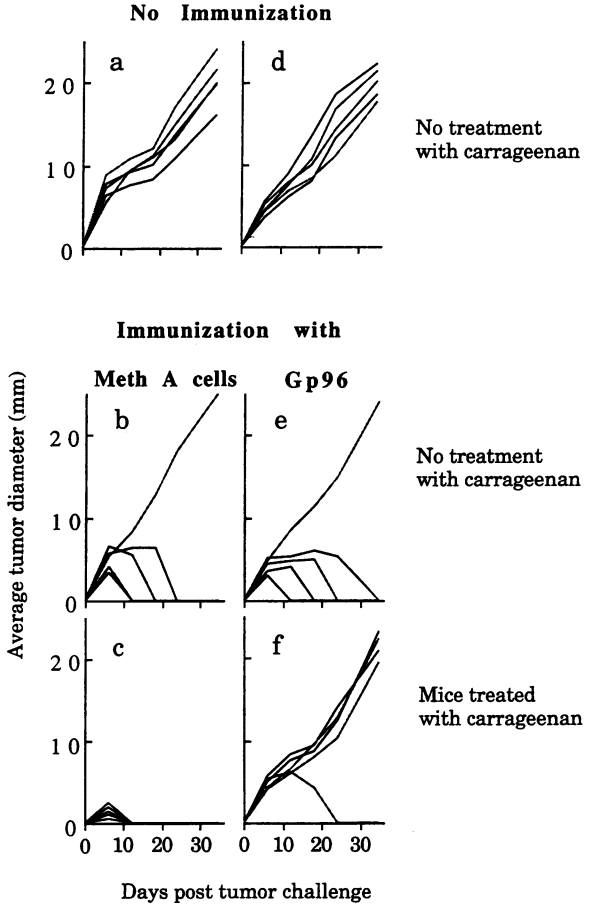

FIG. 5. Requirement of macrophages during the priming phase of tumor immunity generated by vaccination of mice with irradiated Meth A cells (*b* and *c*) or with Meth A-derived gp96 (*e* and *f*). Tumor growth in two independent groups of unimmunized mice is shown (*a* and *d*). Immunized mice were (*c* and *f*) or were not (*b* and *e*) treated with carrageenan. Other details are the same as in legend to Fig. 4. An identical pattern of macrophage requirement was seen in the effector phase.

Fig. 5 shows the results of studies in mice depleted of macrophages by treatment with carrageenan. It is observed that mice treated with carrageenan and immunized with gp96 remain sensitive to Meth A challenge. In contrast, mice treated with carrageenan and vaccinated with intact Meth A cells are still able to effectively resist a Meth A challenge. As macrophage independence of priming of anti-tumor immune response elicited by immunization with intact cells was surprising, we tested it further by multiple carrageenan treatments of mice immunized with intact cells. It was observed that even after treatment with carrageenan every 2 days in the priming phase, mice immunized with intact Meth A cells remained tumor resistant (data not shown).

Our results indicate (see Table 1) that the priming phase requirements of CD4+ and CD8+ T cells and of macrophages

Table 1. Cell types required for tumor immunity elicited by immunization with intact Meth A cells or Meth A-derived gp96

Immunization	CD4+ cells	CD8+ cells	Macrophages
Priming phase			
Intact Meth A cells	R	NR	NR
Meth A-derived gp96	NR	R	R
Effector phase			
Intact Meth A cells	R	R	NR
Meth A-derived gp96	R	R	R

R, required; NR, not required.

in the case of immunization with gp96 are completely opposite to corresponding requirements in the case of immunization with intact cells.

Cellular Requirements in the Effector Phase. The protocol shown in Fig. 3 was followed for determining the requirements for $CD4^+$ or $CD8^+$ T cells or macrophages in the effector phase of tumor immunity. As the effector phase is the latter of the two phases, the precautions regarding the relative timing of immunization, depletion, and tumor challenge used in the priming phase (see previous section) were not necessary. It was observed that depletion of either of the two T-cell subsets in the effector phase abrogated tumor immunity elicited by immunization with intact cells or with gp96 (data not shown). However, a distinction between the two methods of immunization was seen with respect to depletion of macrophages in the effector phase. Similar to the results in the priming phase (Fig. 5), gp96-immunized mice treated with carrageenan immediately prior to tumor challenge were sensitive to a Meth A challenge. In contrast, mice immunized with intact Meth A cells and treated with carrageenan in the effector phase were tumor resistant (data not shown). These results are summarized in Table 1.

DISCUSSION

We observe that (*i*) immunization with gp96 elicits long-lasting tumor immunity, (*ii*) the priming and effector phase events generated by vaccination with gp96 are distinct from the corresponding events elicited by vaccination with intact tumor cells, and (*iii*) immunization with soluble gp96 preparations leads to priming of $CD8^+$ T cells, in spite of the exogenous mode of administration of gp96. This observation points to existence of an additional mechanism of presentation of exogenous immunogens. (*iv*) Immunity to tumors can be arrived at by a macrophage-dependent and a macrophage-independent pathway.

It is instructive to reconstruct a probable chain of events elicited by immunization of mice with intact tumor cells or with tumor-derived gp96. In case of immunization with intact tumor cells, a $CD4^+$ T-cell response is elicited in the priming phase. This can occur in the absence of macrophages, indicating that antigen-presenting cells other than macrophages (such as B cells), which are not depleted by treatment with carrageenan (12, 13), process the tumor antigens and present them to the $CD4^+$ T cells. Early priming of $CD8^+$ T cells is not required. However, the $CD4^+$ and $CD8^+$ T cells are required for tumor rejection after the mice are challenged with live tumor cells. Presumably, the $CD8^+$ T cells are necessary for lysing tumor cells, whereas the $CD4^+$ cells are required to sustain the $CD8^+$ cells by supplying the lymphokines necessary for proliferation of the $CD8^+$ cells.

In case of immunization with gp96, priming of $CD8^+$ but not $CD4^+$ cells appears to be among the earliest events, and it is dependent on the presence of macrophages. Immunization with soluble proteins generally leads to a $CD4^+$ rather than a $CD8^+$ response, because of the different routes of antigen presentation followed by exogenous and endogenously synthesized antigens (14). Soluble gp96 preparations elicit a $CD8^+$ T-cell response in spite of exogenous administration because gp96 is not antigenic *per se* but chaperones antigenic peptides. In light of the macrophage dependence of this $CD8^+$ response, we suggest that gp96–peptide complexes are targeted preferentially to macrophages (as opposed to other antigen-presenting cells), where the peptides are transferred from gp96 to the major histocompatibility complex class I molecules of the macrophage and are able to prime $CD8^+$ responses (15). The ability of macrophages to present exogenous peptides in context of their major histocompatibility complex class I molecules has recently been demonstrated by Pfeifer *et al.* (16) and Kovacsovics-Bankowski *et al.* (17). Both $CD4^+$ and $CD8^+$ cells are required in the effector phase in the case of immunization with gp96 as also in the case of immunization with intact cells. However, in the case of immunity elicited by gp96, there is an additional requirement for macrophages. Most likely, this derives from the requirement for $CD4^+$ cells, which have to be newly and rapidly primed by the macrophage after tumor challenge.

Our results with whole-cell immunization (Fig. 4*c*) imply that $CD8^+$ cells cannot be primed in the absence of a preexisting $CD4^+$ response. This is consonant with the demonstration that stimulation of $CD8^+$ cells in the absence of exogenous help leads to anergy of $CD8^+$ cells (18, 19). It is interesting in this regard that we observe that mice immunized with intact cells but depleted of $CD4^+$ cells (Fig. 4*c*) during the priming phase indeed show enhanced tumor growth as compared to unimmunized mice (Fig. 4*a*). In contrast to the requirement of $CD4^+$ cells for successful priming of $CD8^+$ response in case of whole-cell immunization, we show in Fig. 4*g* that immunization with gp96 leads to priming of $CD8^+$ cells in the absence of $CD4^+$ cells. We suggest that costimulatory molecules such as B7 and CTL4A (20, 21), which are present on macrophages but not on tumor cells, are responsible for this important difference between immunization with whole cells and with gp96. Costimulatory signals can substitute for exogenous help and can permit priming of effective $CD8^+$ T cells (22).

Cellular requirements for tumor immunity have been examined by a number of previous workers. To the extent that the design of our study overlaps with previous studies (i.e., in the effector phase), our results are consonant with and expand the conclusions derived by previous workers (23–25). However, to our knowledge, this is the first report that examines these requirements specifically in the priming and effector phases of the anti-cancer immune response to a solid tumor and shows the presence of a macrophage-dependent and a macrophage-independent pathway of tumor immunity. The observed ability of gp96 molecules to elicit a $CD8^+$ response in the present model as well as in virus-infected cells (26) makes immunization with heat shock proteins an attractive method of vaccination against cancer and infectious diseases.

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