

The Effect of Antigen Dose on CD4⁺ T Helper Cell Phenotype Development in a T Cell Receptor- $\alpha\beta$ -transgenic Model

By Nancy A. Hosken,* Kazuko Shibuya,* Andrew W. Heath,‡
Kenneth M. Murphy,§ and Anne O'Garra*

*From the *Department of Immunology, DNAX Research Institute, Palo Alto, California 94304;*

‡Department of Experimental and Clinical Microbiology, University of Sheffield Medical School,

Sheffield S10 2RX, United Kingdom; and §Department of Pathology, Washington University School of Medicine, St. Louis, Missouri 63110.

Summary

The dose of foreign antigen can influence whether a cell-mediated or humoral class of immune response is elicited, and this may be largely accounted for by the development of CD4⁺ T helper cells (Th) producing distinct sets of cytokines. The ability of antigen dose to direct the development of a Th1 or Th2 phenotype from naive CD4⁺ T cells, however, has not been demonstrated. In this report, we show that the antigen dose used in primary cultures could directly affect Th phenotype development from naive DO11.10 TCR- $\alpha\beta$ -transgenic CD4⁺ T cells when dendritic cells or activated B cells were used as the antigen-presenting cells. Consistent with our previous findings, midrange peptide doses (0.3–0.6 μ M) directed the development of Th0/Th1-like cells, which produced moderate amounts of interferon γ (IFN- γ). As the peptide dose was increased, development of Th1-like cells producing increased amounts of IFN- γ was initially observed. At very high (>10 μ M) and very low (<0.05 μ M) doses of antigenic peptide, however, a dramatic switch to development of Th2-like cells that produced increasing amounts of interleukin 4 (IL-4) and diminishing levels of IFN- γ was observed. This was true even when highly purified naive, high buoyant density CD4⁺ LECAM-1^{hi} T cells were used, ruling out a possible contribution from contaminating “memory” phenotype CD4⁺ T cells. Neutralizing anti-IL-4 antibodies completely inhibited the development of this Th2-like phenotype at both high and low antigen doses, demonstrating a requirement for endogenous IL-4. Our findings suggest that the antigen dose may affect the levels of endogenous cytokines such as IL-4 in primary cultures, resulting in the development of distinct Th cell phenotypes.

The class of immune response (1) that develops upon exposure to foreign antigen has been attributed to heterogeneity in the phenotype (Th1 or Th2) of the responder CD4⁺ T cells (2). Th1 cells are important for cell-mediated immune responses, and they produce cytokines such as IFN- γ and lymphotoxin, which are required for the eradication of viruses and intracellular pathogens (2, 3). Conversely, Th2 cells are associated with allergic responses (2, 3), are important for humoral responses (2, 3), and can regulate Th1-type responses by their production of IL-4 and IL-10 (2–7).

Many studies have indicated that different microbes or protein antigens, when administered using different conditions, routes, or doses, appear to favor the selective induction of cell-mediated or humoral immune responses (1). Although the dose of antigen has been shown to influence both the class of immune response (1, 8, 9) and the produc-

tion of cytokines by differentiated T cells (10–12), the mechanisms by which this may occur are poorly understood. In addition, a direct effect of antigen dose on the development of Th1 or Th2 cells from naive CD4⁺ T cells has thus far not been demonstrated.

Many recent studies, both in vitro and in vivo, have indicated that cytokines present during early T cell activation can determine which Th phenotype develops in response to antigen (13). IL-12, produced by macrophages and dendritic cells (14–16), can direct the development of Th1 cells, which produce large amounts of IFN- γ (17–19). IL-4, which is produced by T cells, is necessary for the development of Th2 cells from naive CD4⁺ T cells (6, 20–22). Thus, by favoring the production of particular cytokines from the APC/accessory cell or T cells present during early T cell activation, various factors may influence Th phenotype development.

In this paper, we have used CD4⁺ T cells from the DO11.10 TCR- $\alpha\beta$ -transgenic mouse to study whether there is a direct effect of antigen dose on Th cell phenotype development. We demonstrate that the development of a Th1- or Th2-like phenotype from naive CD4⁺ T cells is strictly dependent on the dose of antigen used in primary cultures.

Materials and Methods

Animals. Mice transgenic for the OVA₃₂₃₋₃₃₉-specific and I-A^d-restricted DO11.10 TCR- $\alpha\beta$ on a BALB/c genetic background (23) were bred at DNAX. Mice heterozygous for both the TCR- α and - β transgenes were used for experiments. 6–8-wk-old female BALB/c mice were purchased from Simonsen Labs (Gilroy, CA).

Culture Medium, Antigen, and Antibodies. Complete RPMI 1640 (RP10+) and Dutch-modified RPMI 1640 (GIBCO Life Technologies Ltd., Paisley, Scotland) were as previously described (16).

Endotoxin-free OVA peptide (OVA₃₂₃₋₃₃₉; 99% pure) was from Biosynthesis (Lewisville, TX).

Purified 11B11 rat anti-mouse IL-4 mAb (24) and the isotype-matched control rat anti- β -galactosidase mAb GL113 (25) were supplied by J. Abrams (DNAX). Mouse cell-surface antigen-specific mAbs used for flow cytometric sorting or analysis included FITC-conjugated anti-Mac-1 (M1/70; Caltag, San Francisco, CA), biotinylated hamster anti-CD11c (N418 hybridoma from American Type Culture Collection, Rockville, MD; 26); PE-conjugated anti-B220 (RA3-6B2) and anti-CD4 (RM4-5), FITC-conjugated anti-CD3 (145-2C11), CD11a (2D7), CD62L (LECAM-1), and purified anti-CD32/CD16 (24G2) (Pharmingen, San Diego, CA.). Rat anti-CD8 α (53-6-7), I-A^d, B220 (RA3-6B2), and Mac-1 (M1/70) antibodies (either biotinylated or not; Pharmingen). Anti-CD4 mAb RL-172 (American Type Culture Collection) and anti-Thy-1.2 mAb (Accurate Chemical Co., Westbury, NY) were used with complement (Low-Tox MTM; Accurate Chemical) for the lysis of T cells. Additional mAbs for ELISAs, including anti-mouse IL-4 and IFN- γ reagents, were purified from serum-free hybridoma supernatants as previously described (25).

Preparation of TCR- $\alpha\beta$ -transgenic CD4⁺ T Cells. For most experiments, CD4⁺ T cells were purified to >99% homogeneity by flow cytometry, on the basis of staining CD4⁺ or CD4⁺-LECAM-1^{hi}, from the spleen cells of unimmunized TCR- $\alpha\beta$ -transgenic mice as previously described (16). In some experiments, high buoyant density T cells were further selected on Percoll gradients (65–80% interface) before purification by flow cytometry as previously described (16, 27).

Preparation of APCs. Splenic dendritic cells were enriched and then further purified to >98% homogeneity on a FAC-Star^{Plus}® (Becton Dickinson & Co., Mountain View, CA) on the basis of N418^{hi} (CD11c), Mac-1^{lo} staining as previously described (16, 28).

High buoyant density B cells were prepared from the spleens of BALB/c mice as previously described (5) and activated with mitogens for 40 h at 37°C before purification by flow cytometry. Mitogens included 50 μ g/ml polyclonal goat anti-IgM F(ab)₂ antibody fragments (Organon Teknika Corp., Durham, NC), a 1:100 dilution of rat anti-mouse CD40 mAb purified from serum-free hybridoma supernatant (29), or 50 μ g/ml LPS (*Salmo-*

nella typhosa; Sigma Immunochemicals, St. Louis, MO). Viable B cell blasts were collected at the end of the culture period by flotation on Lympholyte MTM (Accurate Chemical) Ficoll gradients, purified by flow cytometry (5) to >99% purity on the basis of staining B220⁺Mac-1⁻CD3⁻, and then viable B cell blasts were again collected on Ficoll as above.

Stimulation of Transgenic CD4⁺ T cells for Cytokine Production. CD4⁺ T cells (2.5×10^5 /well) were stimulated in 2 ml RP10+ in 24-well plates with OVA₃₂₃₋₃₃₉ peptide (100–0.01 μ M) and either B cells (2×10^5 /well) or dendritic cells (2×10^4 /well) (irradiated with 950 R). Some cultures received anti-IL-4 or control mAb (10 μ g/ml). Supernatants were collected at 24 h for determination of IL-2 content by ELISA. Cultures were expanded threefold into fresh medium containing the same concentrations of mAbs at 72 h. T cell blasts (2.5×10^5) were harvested on day 6, washed three times, counted, and restimulated with 5×10^6 irradiated BALB/c splenocytes and 0.6 μ M OVA₃₂₃₋₃₃₉ peptide. Supernatants were collected at 48 h for determination of IFN- γ and IL-4 content by ELISA (25). The lower limit of sensitivity for each of the ELISAs was 125 pg/ml (IFN- γ), 150 pg/ml (IL-4), and 195 pg/ml (IL-2).

Results and Discussion

Antigen Dose Can Direct Differential Th Phenotype Development Irrespective of the Type of APCs Used. The dose of antigen has previously been shown to influence whether a cell-mediated or humoral immune response develops in response to foreign antigen (1, 8, 9). This has been attributed to heterogeneity in the phenotype (Th1 or Th2) of the responder CD4⁺ T cells (2, 3); however, a direct effect of antigen dose on Th phenotype development has not been demonstrated. The studies that we and others have published to date have used a fixed antigen dose while varying the type of APC to study differences in the ability of APC to direct development of discrete Th subsets. To address the possible contribution of antigen dose in influencing Th development, total CD4⁺ T cells from the DO11.10 TCR- $\alpha\beta$ -transgenic mice were allowed to develop in the presence of varying doses of OVA₃₂₃₋₃₃₉ peptide (100–0.01 μ M) during the primary culture period and then were restimulated with a fixed peptide dose (0.6 μ M). T cells from primary cultures containing midrange doses of peptide (0.3–0.6 μ M) produced moderate amounts of IFN- γ and very low levels of IL-4 upon restimulation (Fig. 1), consistent with our previous findings in the DO11.10 system (6, 16, 17). This was true regardless of whether B cells or dendritic cells were used as APC in primary cultures (Fig. 1). Addition of increasing doses of peptide to the primary cultures initially resulted in the development of Th1-like cells that produced increasing amounts of IFN- γ and diminishing amounts of IL-4. The use of very high doses of peptide (>10 μ M), however, resulted in the development of cells with a Th2-like phenotype that produced dramatically larger amounts of IL-4 and lower amounts of IFN- γ . This overall trend of Th phenotype development at different peptide doses was reproducible; however, slight shifts in the absolute dose of peptide required for the development of cells producing particular amounts of IFN- γ and IL-4 were

observed in independent experiments. Increasing numbers of viable T cells were recovered from primary cultures as peptide dose increased, except at the highest peptide concentrations, where cell recoveries declined and an increase in the proportion of dead cells was often observed. Recovery of viable T cells always required the presence of the OVA₃₂₃₋₃₃₉ peptide; no viable cells were recovered when peptide was absent from the primary cultures.

The mechanisms responsible for differential Th phenotype development in response to antigen dose are unclear. Possible explanations include (a) differential outgrowth of Th cells of a given phenotype; (b) differential achievement of threshold levels of endogenous cytokines known to be important for Th cell development; and (c) transmission of qualitatively different signals to individual T cells at different antigen doses. We thus proceeded to dissect these mechanisms further.

Th2 Development at High Antigen Doses Does Not Require the Presence of T Cells Having a Memory Phenotype. The development of a Th2 phenotype at high antigen doses could result from increased production of endogenous IL-4 by the CD4⁺LECAM-1^{lo} subset contained in the total CD4⁺ T cell population. This CD4⁺ T cell subset has previously been shown to be a source of significant amounts of IL-4 in primary cultures and thereby capable of influencing Th phenotype development and directing the development of Th2 cells (16, 27, 30). This T cell subset constitutes ~5%

of the flow cytometry-purified total CD4⁺ T cells used for the antigen-dose experiment shown in Fig. 1 (data not shown). To address the possible contribution of CD4⁺LECAM-1^{lo} T cells, we positively sorted by flow cytometry for CD4⁺LECAM-1^{hi} T cells and tested the effect of antigen dose on Th phenotype development by these cells. Surprisingly, the naive CD4⁺LECAM-1^{hi} T cells also developed a strong Th2 phenotype at high peptide doses, demonstrating that the presence of CD4⁺LECAM-1^{lo} T cells was not required for Th2 development to occur (Fig. 2). B cells stimulated with either anti-IgM (Fig. 2) or anti-IgM, LPS, and anti-CD40 in combination (Fig. 2) directed a similar pattern of Th development across the range of antigen doses. The recovery of T cells varied with the type of B cell APC used (data not shown), however, suggesting that their potency as APC was not a major factor in determining the Th phenotype developed. Of interest was the development of T cells capable of producing significant

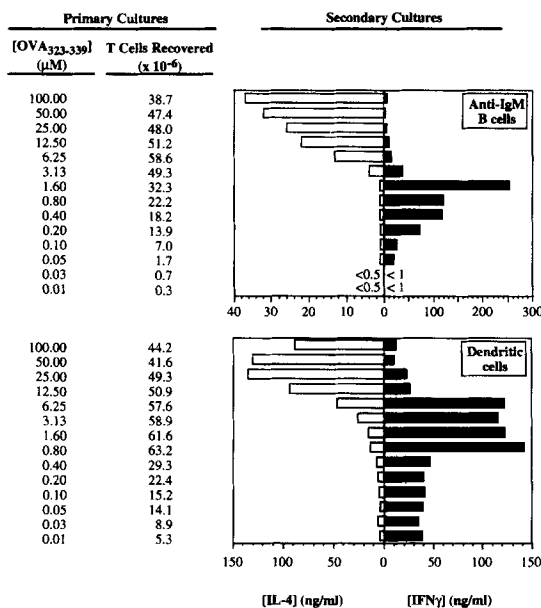


Figure 1. Development of a Th1 or Th2 phenotype is dependent on the antigen dose used in primary cultures. Flow cytometry-sorted, TCR- $\alpha\beta$ -transgenic, CD4⁺ T cells (2.5×10^5 /well) were cultured with flow cytometry-sorted dendritic cells (2×10^4 /well) or activated B cells (2×10^5 /well) and OVA₃₂₃₋₃₃₉ peptide (100–0.01 μ M) in wells of 24-well plates. T cell blasts recovered after 6 d of culture were harvested, washed, and restimulated (2.5×10^5 /well) with BALB/c splenocytes and 0.6 μ M OVA₃₂₃₋₃₃₉ peptide. Supernatants were harvested from secondary cultures at 48 h and tested for IFN- γ and IL-4 content by ELISA. The results shown are representative of three independent experiments.

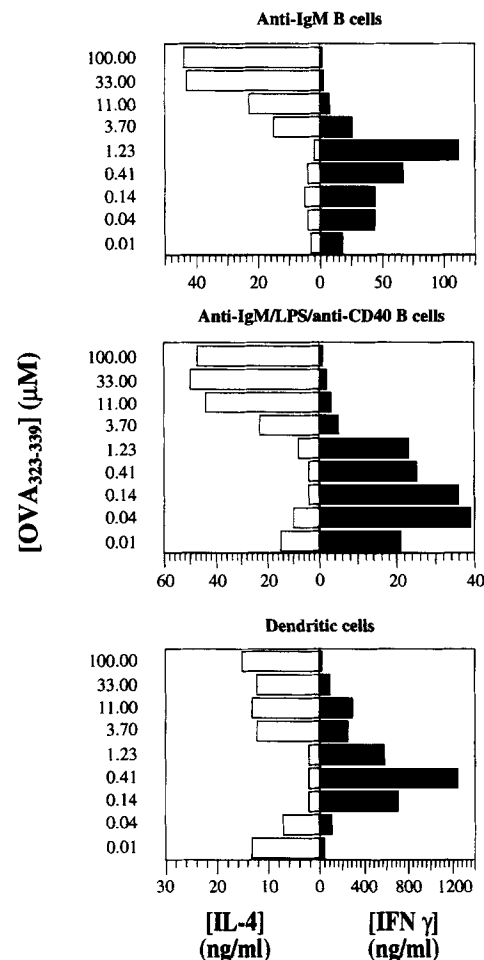


Figure 2. Development of a Th2 phenotype at high antigen doses occurs in the absence of LECAM-1^{lo} CD4⁺ T cells. Flow cytometry-sorted, TCR- $\alpha\beta$ -transgenic, CD4⁺LECAM-1^{hi} T cells were cultured with dendritic cells or activated B cells and OVA₃₂₃₋₃₃₉ peptide for 6 d, harvested, restimulated, and the cytokine content of 48-h supernatants was determined as described in Fig. 1. The results shown are representative of two independent experiments.

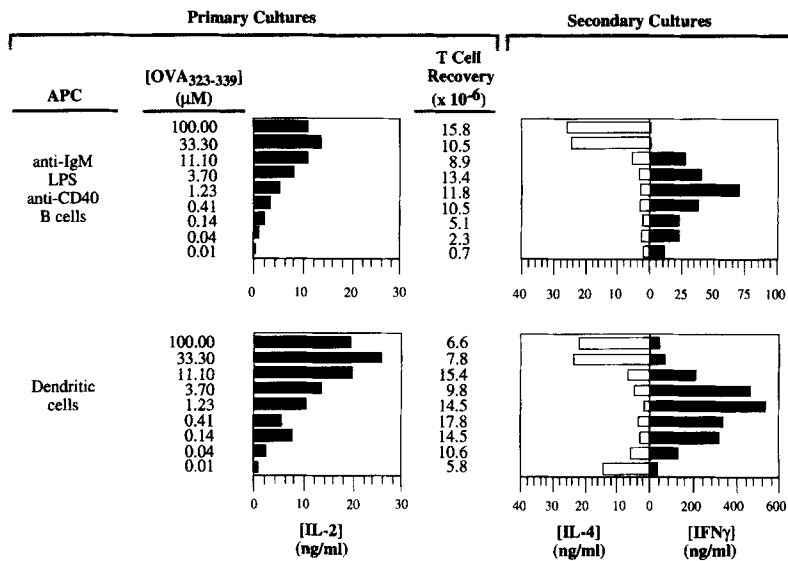


Figure 3. High buoyant density, LECAM-1^{hi} CD4⁺ T cells develop a Th2 phenotype at high antigen doses. Flow cytometry-sorted, TCR- $\alpha\beta$ -transgenic, high buoyant density, LECAM-1^{hi} CD4⁺ T cells were cultured with dendritic cells or activated B cells and OVA₃₂₃₋₃₃₉ peptide for 6 d, harvested, restimulated, and the cytokine content of 24-h supernatants from primary cultures and 48-h supernatants from secondary cultures were determined as described in Fig. 1. The results shown are representative of two independent experiments.

levels of IL-4 at the lowest peptide doses (Fig. 2), which was not seen when total CD4⁺ T cells were used (Fig. 1). This development of IL-4-producing T cells at very low antigen doses coincided with very poor recoveries of T cells from primary cultures. This suggests that there may be preferential survival of a subset of T cells within the initial CD4⁺ LECAM-1^{hi} T cell population that are capable of producing IL-4 upon restimulation.

The possibility that CD4⁺ LECAM-1^{hi} T cells might still contain some T cells precommitted to IL-4 production led us to make further efforts to enrich for naive CD4⁺ T cells. To do this, we purified high buoyant density cells from TCR- $\alpha\beta$ -transgenic spleens on Percoll step gradients before sorting for CD4⁺ LECAM-1^{hi} T cells by flow cytometry. These T cells were then cultured with either activated B cell or dendritic cell APC and varying doses of peptide. The pattern of increasing T cell recoveries from primary cultures as antigen dose increased (Fig. 3) was similar to that seen in previous experiments (Figs. 1 and 2), and we reasoned that this might result from limiting amounts of IL-2 being produced by the T cells at low antigen doses. This was supported by finding that the IL-2 content of supernatants taken at 24 h from primary cultures was low to undetectable at the lowest peptide doses and rose to a plateau as peptide dose increased (Fig. 3). Addition of rIL-2 (500 U/ml) to primary cultures of naive CD4⁺ LECAM-1^{hi} T cells with dendritic cell APC, however, did not significantly increase T cell recoveries or alter the Th phenotype developed at any peptide dose (data not shown). The pattern of Th phenotypes developed from the high buoyant density CD4⁺ LECAM-1^{hi} T cells on the different types of APC was essentially identical to that previously seen from CD4⁺ LECAM-1^{hi} T cells, with median peptide doses (1 μ M) giving rise to Th1-like cells producing IFN- γ and very high (>10 μ M) and low (<0.05 μ M) peptide doses driving the development of Th2-like cells producing IL-4 (compare Figs. 2 and 3).

Th2 Phenotype Development at High and Low Antigen Doses Is Dependent on Endogenous IL-4.

IL-4 has been shown to be the dominant cytokine required for development of a Th2 phenotype from naive CD4⁺ T cells. To determine whether Th2 development at high antigen doses in our system was dependent on endogenous IL-4, anti-IL-4 mAb was added to primary cultures of naive CD4⁺ LECAM-1^{hi} T cells containing different doses of OVA₃₂₃₋₃₃₉. The anti-IL-4 mAb strongly inhibited Th2 development at both high (100 μ M) and low (<0.01 μ M) peptide doses, whereas the isotype control antibody had no effect (Fig. 4). These data clearly demonstrate that the development of Th2-like cells resulting from the addition of very high doses of peptide in primary cultures is IL-4 dependent. This was true both when dendritic cells and anti-IgM/LPS/anti-CD40-activated B cells were used as the APC

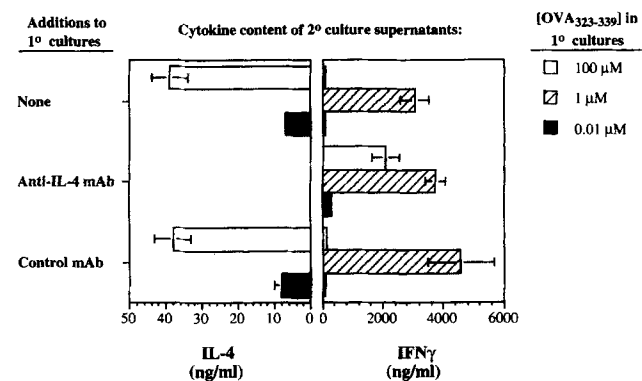


Figure 4. Endogenous IL-4 is required for Th2 phenotype development at high antigen doses. Flow cytometry-sorted, TCR- $\alpha\beta$ -transgenic, LECAM-1^{hi} CD4⁺ T cells were cultured with dendritic cells and OVA₃₂₃₋₃₃₉ peptide (100, 1, or 0.01 μ M) for 6 d, harvested, restimulated, and the cytokine content of 48-h supernatants were determined as described in Fig. 1. The primary cultures contained either anti-IL-4 mAb or isotype control mAb (10 μ g/ml) where indicated. The results shown are representative of three independent experiments.

(Fig. 4 and data not shown), neither of which have been described to produce IL-4. This suggests that the high buoyant density LECAM-1^{hi} population of CD4⁺ T cells may be heterogeneous and contain a subpopulation of cells capable of producing significant amounts of IL-4 upon primary stimulation with high doses of antigen in vitro. Alternatively, this population of T cells may be homogeneous and capable of producing sufficient IL-4 to direct Th2 development. The dependence of Th2 development at low doses of antigen is surprising, given that sufficient IL-4 to direct Th2 development is unlikely to be produced. The Th2 cells recovered from these cultures may instead reflect preferential survival of previously activated T cells having the capacity to produce IL-4.

Our data are the first to show that the dose of antigen used to stimulate naive CD4⁺ T cells can dictate the development of a Th1- or Th2-like phenotype. Furthermore, the development of Th2-like cells from naive CD4⁺ T cells stimulated with very high or low doses of antigen is

IL-4 dependent. A default pathway of Th development may normally operate to produce an appropriate balance of cell-mediated and humoral immunity for clearance of pathogens. The nature of this response may be determined by the genetic background of the host as well as the prevailing immune milieu at the time of antigen exposure. This developmental pathway can clearly be overridden, however, to obtain a higher degree of cell-mediated immunity by activation of macrophage/monocyte-lineage cells to produce IL-12 (14–19). Conversely, immunization with antigen in a manner that does not activate such cells, but induces sufficient production of endogenous IL-4 by CD4⁺ T cells (e.g., high dose soluble antigen), may shift the balance towards stronger humoral immunity by inducing the development of T cells producing significant levels of IL-4. Thus, by affecting the balance of cytokine production by the APC/accessory cells or T cells, a variety of factors, including antigen dose, may determine the class and strength of the immune response to foreign antigens.

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Address correspondence to Dr. Nancy Hosken, DNAX Research Institute, 901 California Avenue, Palo Alto, CA 94304-1104.

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