Retinoids Downregulate Both p60 and p80 Forms of Tumor Necrosis Factor Receptors in Human Histiocytic Lymphoma U-937 Cells

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Because retinoids are known to modulate the growth and differentiation effects of tumor necrosis factor (TNF), we investigated the effect of all-trans-retinoic acid (RA) on the cell surface expression of TNF receptors in human histiocytic lymphoma U-937 cells. RA decreased the specific binding of [125I]-labeled TNF to these cells in a dose- and time-dependent manner. The maximal decrease occurred when cells were treated with 1 μmol/L RA for 24 hours at 37°C. Scatchard analysis of the binding indicated that the decrease by RA was caused by a decrease in receptor number and not by a decrease in affinity. The downmodulation of TNF receptors was also confirmed by covalent receptor-ligand cross-linking studies. Receptor-mediated internalization of the ligand was also found to be decreased on treatment of cells with RA. Northern blot analysis also indicated a decrease in the transcript of the receptor. By using antibodies specific to either the p60 or p80 form of the TNF receptor, we found that both receptors were downregulated by RA. RA treatment also decreased TNF receptors on acute monocytic leukemia cell line THP-1. Other analogues of RA, specifically 9-cis-RA, (E)-4-[2-(5,6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl]-benzoic acid (TTNPB), and 3-methyl-TTNPB, which differ in their specificity towards different RA receptors, were also active in downregulating TNF receptors. 3-Methyl-TTNPB, which is more specific for the RXR form of the RA receptor, was found to be most potent. The downregulation of TNF receptors by RA correlated with the downmodulation of the antiproliferative effects of TNF against U-937 cells. Overall, our results indicate that RA downmodulates both the p60 and p80 form of the TNF receptor on cells of myeloid origin, which correlates with the cellular response.

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TUMOR NECROSIS FACTOR (TNF), a cytokine produced primarily by activated macrophages, plays an important role in cancer, cachexia, septic shock, immunomodulation, inflammation, and differentiation. TNF exerts these multiple effects by binding to specific receptors on target cells through two different TNF receptors with molecular masses of about 60 kD (p60) and 80 kD (p80). Although most cells express both receptors, their relative abundance varies among different cell types. The p60 form of the TNF receptor is more prevalent on epithelial cells, whereas the p80 receptor is more abundant on cells of myeloid origin. The cell surface expression of TNF receptors is upregulated by interferons, dibutyryl cAMP, butyrate, TNF, thyroid-stimulating hormone, and lectins, whereas it is downregulated by interleukin-1 (IL-1), granulocyte-macrophage colony-stimulating factor, phorbol esters, glactocorticoids, lipopolysaccharides, and ocadac acid. Phosphatases and kinases play a role in the regulation of TNF receptors.

Retinoids (natural and synthetic analogues of vitamin A) have been shown to modulate the receptors for number of different cytokines. They enhance the expression of receptors for IL-1, IL-2, transforming growth factor (TGF) β1, epidermal growth factor (EGF), TNF, and nerve growth factor (NGF), but decrease the receptors for autocrine motility factor (AMF) in melanoma cells, IL-6 receptors in myeloma, and EGF receptors in cervical carcinoma ME-180 cells. Retinoids modulate the growth and differentiation of both myeloid and epithelial cells in vitro and display anti-inflammatory properties in vivo. They have been introduced as therapy for acute promyelocytic leukemia in clinical studies. In combination with various cytokines, all-trans-retinoic acid (RA) shows a synergistic activity for differentiation of hematopoietic cells, but the mechanism by which they synergize is not understood.

TNF has been shown to synergize with RA for differentiation of different cell types. Both TNF and retinoids are also potent modulators of proliferation of different tumor cells. In addition, we have recently shown that lipopolysaccharide- and interferon (IFN) -γ-stimulated TNF production by macrophages is inhibited by RA. In the present study, we investigated the effect of RA on the expression of TNF receptors in U-937 cells. Our results indicate that both types of TNF receptor are downregulated by RA in U-937 cells and that this correlates with the cellular response. We also show that RA analogues that have specificity towards different classes of receptors are active in the modulation of TNF receptors.
Dr Peter Davis (University of Texas Health Science Center, Houston, TX). (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) was from Promega (Madison, WI).

*Cell culture.* U-937 (histiocytic lymphoma, CRL 1593) and THP-1 (acute monocytic leukemia, TIB 202) cell lines used in this study were obtained from American Type Cell Culture Collection (Rockville, MD). These cells were grown in RPMI-1640 supplemented with 10% FBS and 50 μg/mL gentamicin.

**RA treatment.** Solutions of RA (1 mmol/L), retinol (1 mmol/L), 9-cis RA (5 mmol/L), TTNBP (5 mmol/L), and 3-methyl-t-TTNBP (5 mmol/L) were prepared in dimethyl sulfoxide (DMSO) and then diluted in the appropriate medium. Cells (0.2 to 0.5 x 10^6/mL) were incubated with RA in a serum-free RPMI-1640 medium supplemented with 50 μg/mL gentamicin at 37°C for 0 to 24 hours in 12-well plates or in T25 tissue culture flasks. The medium was then removed, and the cells were washed, counted, for viability, and then examined for TNF receptors. An appropriate DMSO control was run wherever necessary.

**Receptor binding assays.** Receptor binding assays were performed as described previously.2,4,15 TNF was labeled with Na''1 using the iodogen method.4 Briefly, 10 μg of TNF in a 20-μL volume was placed onto a film of 50 pg of Iodogene and incubated for 10 minutes at 4°C in the presence of 1 mL of carrier-free Na''1. Free iodine was removed by gel filtration on a PD-10 column equilibrated with phosphate-buffered saline (PBS) containing 0.1% gelatin. More than 96% of the iodine in the final product was incorporated in the protein as determined by trichloroacetic acid (TCA) precipitation. The specific activity of the labeled TNF ranged from 20 to 30 mCi/mg.

Binding assays were performed in flexible 96-well plates pre-coated with 0.2 mL of FBS for 24 hours at 4°C. The binding medium (RPMI-1640) contained 10% FBS. Cells (0.4 to 0.5 x 10^6/mL) were incubated with 1''1-labeled TNF in the absence (total binding) or in the presence of 100 μmol/L unlabeled ligand (nonspecific binding) for 1 hour at 4°C. The cells were washed three times with ice-cold medium (PBS containing 0.1% bovine serum albumin [BSA]) at 4°C, and the cell-bound radioactivity was determined in a γ-counter (Cobra-Auto-Gamma; Packard Instrument Co, Meriden, CT). All determinations were performed in triplicate. Specific binding of the 1''1-labeled TNF was calculated by subtraction of nonspecific binding from the total binding. Inhibition of specific binding by RA was calculated from the specific binding obtained from the untreated cells (100%). The dissociation constant (kd) and the number of receptors were calculated by Scatchard analysis as described.4,15

To examine the expression of p60 and p80 form of the TNF receptors, affinity-purified antibodies specific against each receptor were used. Cells (0.5 x 10^7/mL) were preincubated with the antibody (2 μg/mL) for 1 hour at 4°C and then examined for specific binding of labeled TNF as described above. Specific binding of TNF observed on cells pretreated with anti-p60 antibody is due to p80 receptor and that observed with anti-p80 antibody-pretreated cells is due to the p60 receptor.

**Covalent cross-linking of TNF to cell surface receptors.** The cross-linking procedure was performed according to the previously described method.4 Briefly, cells (2 x 10^6) were incubated with 2.5 mmol/L 1''1-labeled TNF in 1 mL RPMI-1640 for 1 hour at 4°C, washed three times with ice-cold PBS to remove unbound ligand, and then treated with 0.1 μmol/L (final concentration) ethylene glycol-bis (succinimidyl succinate) (EGS), the cross-linking reagent. After 45 minutes of incubation at room temperature, EGS was quenched by 0.2 μmol/L sodium carbonate (final concentration). The cells were washed with ice-cold PBS and solubilized with solubilization buffer (50 mmol/L Tris, pH 7.5) supplemented with aprotinin (2 μg/mL), phenylmethylsulfonyl fluoride (1 mmol/L), leupeptin (2 μg/mL), NP40 (0.5%), sodium chloride (200 mmol/L), and β mercaptoethanol (0.1%). After 10 minutes of incubation at 4°C, the samples were centrifuged and the supernatants were analyzed on 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was dried and exposed to a Phosphorimag screen (Molecular Dynamics, Sunnyvale, CA), and the image was recorded and quantitated using "Image Quant" software.

**Receptor-mediated internalization of TNF.** The ligand internalization experiments were performed according to the procedure as described previously.15 Briefly, U937 cells (0.5 x 10^6/mL) were incubated either with or without RA (1 μmol/L) for 24 hours at 37°C in serum-free medium. The cells were then washed and incubated in a fresh medium for 2 hours at 4°C with 0.5 x 10^6 cpm/mL of 1''1-labeled TNF, and then unbound 1''1-TNF was removed by three washes with cold PBS containing 0.1% BSA. The kinetics of internalization of TNF was examined by incubating the cells with fresh medium at 37°C. At indicated times, medium was removed and counted for radioactivity in the gamma counter. The cell-surface bound TNF was eluted from cells by acid washing twice (0.2 mol/L acetic acid and 0.5 mol/L sodium chloride, pH 3.0) at different times and counted. For determination of internalized TNF (the acid-nondissociable fraction), acid-washed cells were solubilized in 0.5 mol/L sodium hydroxide and then counted. The acid wash procedure used to remove the cell surface-bound TNF did not significantly affect cell viability as determined by trypan blue exclusion.

**Shedding of TNF receptor.** U-937 (0.5 x 10^6/mL) cells were incubated with or without RA at 37°C, and culture supernatants were removed after centrifugation. The amount of soluble TNF receptors was assayed by using a TNF receptor enzyme-linked immunological assay (ELISA) kit (Hoffmann-La Roche, Basel, Switzerland; p60 kit no. S-0740 and p80 kit no. S-2140). Briefly, 0.2 mL of monoclonal anti-p60 or anti-p80 receptor antibody (0.5 ng/mL) in 100 mmol/L sodium phosphate, pH 6.5, was added to a 96-well enzyme-linked immunosorbent assay (ELISA) plate (Nunc 406667, Naperville, IL) and incubated at room temperature overnight. Wells were then washed three times with water, and 0.2 mL of 200 mmol/L Tris-HCl, pH 7.5, containing Kathon MW/WT and 0.1% BSA was added. After overnight incubation, wells were again washed three times; test samples or standard solutions of recombinant soluble p60 and p80 were then added. After overnight incubation at room temperature, wells were washed four times with 0.05% Tween 20 in H2O and twice with H2O alone. For detection, 0.2 mL of 30 mmol/L potassium citrate buffer, pH 4.1, containing Kathon MW/WT, 500 mmol/L sodium citrate, 3.3', 5.5'-tetrathymethylenediamine, and 4 mmol/L H2O2 was added and the solution was incubated for 30 minutes. To stop the reaction, 50 μL of 1 mol/L sulfuric acid was added. Absorbance at 450 nm was measured and the amount of p60 and p80 receptor in the test samples was evaluated from the standard curve.

**Northern blot analysis of the expression of p60 and p80 mRNAs.** U-937 cells (0.5 x 10^6/mL) were treated with 1 μmol/L RA for different times. Total cellular RNA was extracted from cells as described previously.15 For Northern blot analysis, RNA samples (20 μg) were denatured with formamide and formaldehyde and electrophoresed in 0.8% agarose gel containing 0.67 mol/L formaldehyde at 75 V for approximately 3 hours. RNA was alkali-transferred to Hybond N+ nylon membrane (Amersham Corp). After alkali transfer, the membrane was rinsed with 2 x SSC (1 x SSC: 0.15 mol/L sodium chloride, 0.015 mol/L sodium citrate, pH 7.0). It was prehybridized at 65°C for 1 hour in hybridization buffer containing 7% SDS and 1 mmol/L EDTA in 0.5 mol/L sodium phosphate buffer, pH 7.2. The membrane was then hybridized for 16 to 20 hours with 3''P-labeled cDNA probes for p60 and p80 (approximate specific activity, 5 to 10 x 10^6 cpm/μg DNA) in the hybridization buffer, plus denatured salmon sperm DNA (0.2 mg/mL). After hybridization, the membrane was extensively washed with 2 x SSC and 0.1% SDS. The membrane was then exposed to a Phosphorimag screen (Molecular Dynamics), and the image was recorded as described.
bridization, the membrane was washed twice with 2X SSPE (1X SSPE: 0.18 mol/L NaCl, 0.01 mol/L sodium phosphate, and 1 mmol/L EDTA) containing 0.1% SDS at room temperature, followed by another wash with 1X SSPE containing 0.1% SDS at 65°C. The blots were then exposed to phosphorimager screen (Molecular Dynamics, Sunnyvale, CA), and the images were recorded and quantitated using "Image Quant" software. To show the equal loading of lanes, the probes were stripped off the membrane by washing the filters twice with 0.5% SDS at 95°C for 30 minutes. The membrane was then hybridized with a cDNA probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Antiproliferative assays. To examine the antiproliferative effects of TNF, U-937 cells (0.5 × 10^6 cells/mL) were treated for 16 hours with RA (1 μmol/L) or DMSO (0.02%) as a control in serum-free RPMI-1640 medium. Subsequently, cells (5 × 10^5 cells/0.2 mL) were washed and plated in a 96-well flat-bottom plate (Falcon cat. no. 3072) in a complete medium containing different concentrations of hTNF. After 72 hours at 37°C, cells were assayed for viability using the Promega CellTiter 96 AQueous Non-Radioactive Proliferation Assay kit (cat. no. G5421). Cell viability was expressed by plotting the absorbance at 490 nm. Antiproliferative effects of TNF were calculated by using following formula:

\[
\text{Antiproliferative Effects (\% of control)} = 100 - \left( \frac{\text{Absorbance With TNF}}{\text{Absorbance Without TNF}} \times 100 \right)
\]

RESULTS

RA downregulates cell surface expression of TNF receptors. To examine the effect of retinoic acid on TNF receptors, U-937 cells were treated with different concentrations of RA at 37°C for 24 hours, washed, and then analyzed for cell surface expression of TNF receptors (Fig 1, upper panel). RA decreased the specific binding of ^125I-labeled TNF on U-937 cells in a dose-dependent manner. The maximum decrease (approximately 50%) in binding was observed when cells were treated with 1 μmol/L RA. This concentration had no effect on the viability of U-937 cells, as determined by cell counting or by ^3H thymidine incorporation (data not shown). The decrease of TNF receptors by RA was also not due to an effect on protein synthesis, as evaluated by ^3H leucine incorporation (data not shown). Next, we studied the time-course of downregulation of TNF receptors on U-937 cells by RA. The cells were exposed to 1 μmol/L of RA at 37°C for different times and then assayed for specific binding of ^125I-TNF. Binding decreased in a time-dependent manner throughout the RA treatment (Fig 1, lower panel). A 58% decrease in binding was observed after 24 hours. When we examined the effect of RA (1 μmol/L for 24 hours) on TNF receptors in acute monocytic leukemia cell line, THP-1, a 56% decrease in specific binding of TNF (1,000 cpm v 342 cpm) was observed.

We used Scatchard analysis to determine the effects of RA treatment on the number and/or binding affinity of TNF receptors. The cells were treated with 1 μmol/L RA for 24 hours at 37°C, washed, and then analyzed for receptor binding characteristics by incubating cells with variable amounts of ^125I-TNF in the absence or presence of a 100-fold excess of unlabeled TNF (Fig 2, upper panel). For untreated cells, the receptor numbers/cell and dissociation constant (kd) were 4,554 sites/cell and 1.0 nmol/L, respectively, but for cells treated with 1 μmol/L RA, the values were 3,044 receptor numbers/cell and 105 nmol/L, respectively. These results indicated that RA decreased the number of TNF receptors, whereas the affinity of the receptors did not change significantly (Fig 2, lower panel).

To determine whether the downmodulation of TNF receptors by RA affects ligand internalization, we examined the rate of internalization and the dissociation of the ligand from
To further confirm the RA-mediated downmodulation of TNF receptors, we performed receptor-ligand cross-linking studies on U-937 cells. $^{125}$I-labeled TNF was cross-linked to the cell surface receptors on control and RA-treated cells using EGS, and the ligand-receptor complexes were visualized after SDS-PAGE (Fig 4). We observed a single band with an approximate molecular mass of 97 kD in untreated U-937 cells. A band with the same molecular mass was also observed in RA-treated cells, but computer-assisted densi-
MODULATION OF TNF RECEPTORS BY RETINOIDS

...complex was Therefore, the downregulation of the cell surface expression of TNF receptors is accompanied by...modulation was complicated whether downregulation of the cell surface expression of TNF receptors induced by RA was...ory' that 60% to 80% of the total TNF-binding sites on U-937 cells can be attributed to the p60 form of the TNF receptor. The results of these experiments, shown in Fig 6, indicate that RA downmodulated both forms of the TNF receptors to a similar extent.

Downmodulation of TNF receptors by RA is not due to its shedding. We also investigated the possibility that RA...decreases the number of cell surface TNF receptors by causing their release (shedding) into the medium. For these experiments, U-937 cells were treated with 1 μmol/L of RA at 37°C for 6 hours and 24 hours. The shed form of the TNF receptor in the culture medium was analyzed by an ELISA kit. RA treatment had no effect on the shedding of the p60 and p80 forms of the receptor (data not shown).

RA analogues also downregulate TNF receptors on U-937 cells. The intracellular effects of RA are mediated by two families of retinoid receptors, RA receptors (RARs) and retinoic X (RXRs) receptors. Whereas all-trans-RA binds to RAR, 9-cis-RA binds with high affinity to the RXR family. Similarly, TTNPB and 3-methyl-TTNPB have higher affinities for RAR and RXR, respectively. To investigate the role of different retinoid receptors in TNF binding, we treated U-937 cells with different retinoid analogues (1 μmol/L) for 24 hours and then examined them for TNF receptors. The results indicate that, under identical conditions, 3-methyl-TTNPB was maximally effective (80%) and retinol was least effective (10%) in decreasing TNF receptors (Fig 7). No significant difference was observed between cis and all-trans forms of RA (45% to 50%), and TTNPB was as effective as all-trans-RA. These observations suggest that both classes of retinoid receptors are involved in the modulation of TNF receptors on U-937 cells.

RA also downregulated the cellular response of TNF in U-937 cells. TNF has been shown to exhibit antitumor effects against U-937 cells. To correlate the decrease of TNF receptors by RA with the TNF-dependent cellular responses, we examined the antiproliferative effects of TNF on treated and untreated U-937 cells. The results shown in Fig 8 indicate that TNF decreases the rate of proliferation of these cells and this decrease is significantly abolished when cells are pretreated with RA. These results thus suggest that decreased TNF receptors by RA could protect the cells from the antiproliferative effect of TNF.

**Fig 5.** Effect of RA on the steady state levels of the mRNA for p60 and p80 forms of the TNF receptor. For each point, 10 x 10^6 U-937 cells at 0.5 x 10^6/ml were incubated either with or without 1 μmol/L retinoic acid for 6 hours and 24 hours at 37°C either in the absence of serum. The total RNA was isolated, electrophoresed, and hybridized with specific probes as described under Materials and Methods. One typical result out of three independent experiments is shown.

**Fig 4.** Effect of RA treatment of the U937 cells on the receptor-ligand cross-linking. Cells (0.5 x 10^6/ml) were incubated either with or without 1 μmol/L RA for 24 hours at 37°C. Cells were then rinsed, performed the receptor binding, cross-linked the receptor ligand complex with EGS, and then analyzed by SDS-PAGE as described under Materials and Methods. One typical result of three independent experiments is shown.
Fig 6. Effects of RA on the cell surface expression of p60 and p80 form of TNF receptors in U-937 cells. Cells (0.5 × 10^6/mL) were incubated with 1 μmol/L RA for 24 hours at 37°C in serum-free medium. Thereafter, the cells were washed and examined for p60 and p80 form of the TNF receptors by using anti-p60 or anti-p80 antibodies as indicated in Materials and Methods. Specific binding of labeled TNF observed on cells pretreated with anti-p60 antibody is due to the p80 receptor and that observed with anti-p80 antibody-pretreated cells is due to the p60 receptor. All determinations were performed in triplicate. The numbers on top are the percentages.

DISCUSSION

In the present report, we show that all-trans-RA decreases the cell surface expression of TNF receptors on U-937 cells. The decrease in TNF receptors by RA was not unique to U-937 cells but was also observed with another myeloid cell line, THP-1. Scatchard analysis showed that RA downregulated TNF receptor number without significantly changing the receptor affinity. Furthermore, both forms of the TNF receptor were downmodulated by RA, which was accompanied with the decrease in the cellular response to TNF. Studies with analogues of RA indicated that the downregulation of TNF receptor was mediated through both RAR and RXR forms of the RA receptor.

Our results indicate that both types of TNF receptor in U-937 cells are downmodulated by RA when treated for 24 hours. In contrast, Winzen et al.24 showed that RA treatment of HL-60 cells increased TNF binding. The reason for this discrepancy is not clear. The treatment of HL-60 cells with RA under our experimental conditions had no effect on TNF receptors (data not shown). This difference may represent a difference in the experimental conditions. For instance, during our experiment, we treated the cells with RA in serum-free medium for 24 hours, whereas they used serum-containing medium and long-term treatment with RA. Despite an increase in binding of TNF by RA on HL-60, no change in the mRNA of the TNF receptor was reported.24 These results are also inconsistent with ours on U-937 cells, in which we showed changes in TNF receptor levels by binding, by receptor-ligand cross-linking and receptor-specific blocking antibodies, and by mRNA analysis. Besides myeloid cell lines, we found the modulation of TNF receptors by RA also in nonmyeloid cell lines such as T-47D (breast carcinoma) and H-596 (lung carcinoma).

As we showed for the modulation of TNF receptors by RA, the receptors for IL-6, AMF, and EGF have also been shown to be downmodulated by RA.22,23-27,51 In contrast, the receptors for IL-2, TGF-β, and NGF are increased by RA.20,21,27 How RA induces the receptors for some cytokines while downmodulating that of others is not clear. Because RA modulates the expression of several different cytokines,20,40,52 it is possible that the expression of cytokines by RA could in turn lead to the modulation of TNF receptors. The cell surface expression of TNF receptors can be downregulated by a number of different cytokines including IL-1, granulocyte monocyte colony-stimulating factor, and TNF.50,53,54 However, it is unlikely that RA-mediated downmodulation of TNF receptor is through the induction of TNF, because the latter has recently been shown to be also downmodulated by RA.40

There are several reports that show that activators of protein kinase C (PKC) downmodulate TNF receptors, and RA has been shown to induce PKC in certain cells55; therefore,
it is possible that the effects of RA on TNF receptors are mediated through the activation of PkC. However, we consider this possibility unlikely, because activation of PkC has been shown to cause the shedding of TNF receptors\(^1\) and no receptor shedding was observed on cells treated with RA. Furthermore, the kinetics of TNF receptor downmodulation by PkC activators was much more rapid than that observed with RA. The intracellular processes by which RA could interfere with the cell surface TNF receptors are probably related to their replacement and/or degradation.\(^5\)\(^6\) Although little is known about the posttranslational processing of TNF receptors, it is clear that posttranslational modifications such as complex glycosylation are important in their final processing. The role of RA in modulating both glycoconjugate biosynthesis\(^7\) and lysosomal activity\(^8\) has been demonstrated. These processes may also modulate TNF receptors.

The regulation of TNF receptor does not always correlate with the cellular response.\(^9\)\(^10\) This relationship varies depending on the nature of the TNF-dependent response and also on the cell line. For instance, antiapoptotic effects of TNF require full receptor occupancy,\(^9\) whereas for TNF-dependent NF-κB activation only 10% to 25% of the total receptors on U-937 cells were found sufficient for full response.\(^9\) Similarly, interferons have been shown to induce TNF receptors,\(^9\)\(^10\) but this induction alone is not sufficient to account for their synergistic cellular response.\(^9\) However, in the present studies, we found that the downregulation of TNF receptors by RA accompanied with a decrease in the antiproliferative effects of TNF. Overall, our results indicate that RA can downmodulate TNF receptors in myeloid cells and this may have a role in well-described modulation of the cytokine responses by RA.

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