Tumor necrosis factor (TNF) can inhibit the differentiation of preadipocytes to adipocytes and will revert differentiated adipocytes to the preadipocyte state. TNF is not toxic to either adipocytes or preadipocytes when used alone but is highly toxic to these cells when used in conjunction with cycloheximide, yielding virtually 100% killing within 4–6 h of treatment. A cell line (TA1 R-6) was isolated which is resistant to the combined toxic effects of TNF and cycloheximide. This cell line is stable and, unlike the parental cell line, does not morphologically differentiate to adipocytes or express adipocyte-specific mRNAs. It has a more transformed appearance and growth pattern and, while resistant to the toxic effects of TNF and cycloheximide in a 6-h assay, has become sensitive to cytotoxicity induced by TNF used alone in a 3-day assay. The adipocyte differentiation-inducing agents, dexamethasone and indomethacin, block the cytotoxicity induced by TNF alone in the TA1 R-6 line but do not block the rapid cytotoxicity of TNF and cycloheximide in the parental line. These results provide both genetic and pharmacologic evidence that there are at least two distinct or overlapping pathways by which TNF mediates its effects.

Tumor necrosis factor (TNF) is a protein that exerts cytotoxic and cytostatic effects against certain tumor cell lines while sparing normal cells both in vitro and in vivo (1-4). The selective action of this monokine against tumor cells appears not to be due to differences in TNF receptors since both sensitive and resistant cells have similar numbers of receptors with equivalent affinities for TNF (5-7). Furthermore, many normal cells exhibit characteristic responses to TNF yet are not growth inhibited. For example, TNF augments the growth of normal diploid fibroblasts (8, 9), activates polymorphonuclear neutrophils (10), induces differentiation of myelogenous leukemia cells into the monocyte/macrophage pathway (11), inhibits the differentiation of muscle cells (12), and inhibits the differentiation of adipocytes (13). This latter activity has been associated with the cachenix activity ascribed to TNF and can be detected by the inhibition of adipose-specific gene expression in adipogenic cell lines such as 3T3-L1 or TA1 (14-17). These findings suggest that the selective toxicity of TNF against certain tumor cell lines and the various other functions of TNF may be mediated by activating complex and perhaps independent biochemical pathways.

To further analyze the mechanisms by which TNF exerts its diverse functions, one would like to have variant cell lines that exhibit altered responses to TNF. We have recently focused our attention on the ability of TNF to inhibit differentiation of adipogenic cell lines, a reflection of its possible role in cachexia. During the course of these studies, TA1 cells were found to be resistant to the cytotoxic action of TNF but rapidly killed when treated simultaneously with TNF and cycloheximide. Cycloheximide has previously been shown to enhance TNF-mediated killing of other cells in culture (18). This observation formed the basis for selecting variant cell lines resistant to the cytotoxic actions of TNF seen in cycloheximide-treated TA1 cells. Among these resistant lines, one exhibited the unusual property of sensitivity to TNF alone in a standard cytotoxicity assay. In this manuscript we describe the characteristics of this variant line derived from TA1 cells and provide both genetic and pharmacological evidence that TNF cytotoxicity in the presence of cycloheximide entails activation of a pathway for cell killing distinct from that involved in standard (3 day) TNF-mediated cytotoxicity. Our results suggest that at least two different signaling pathways stimulated by TNF are associated with cytotoxicity, one of which may also play a role in inhibiting adipocyte differentiation.

MATERIALS AND METHODS

Cell Culture Conditions—TA1, TA1 R-6, and mouse L-929 cells were grown in Eagle’s basal medium (GIBCO) supplemented with 10% heat inactivated (56 °C for 30 min) fetal calf serum. Cultures were grown at 37 °C in a humidified incubator in a 5% CO₂ atmosphere. The medium was changed every 2 days. To accelerate differentiation, confluent plates of cells were treated with indomethacin or dexamethasone as previously described (19, 20). Cells were selected in cycloheximide (10 μg/ml) prepared in phosphate-buffered saline. TNF (Cetus) was reconstituted as directed and stored in frozen aliquots at −70 °C until use. For analysis of cytotoxicity, TA1 cells were plated in 96-well microtiter plates at 1 × 10⁴ cells/well in 100 μl of complete media and allowed to reach confluence prior to treatment.

RNA Isolation and Analysis—Total RNA was prepared by a modification of the method described by Chirgwin et al. (21). Briefly, 10-cm plates were washed with phosphate-buffered saline (4 °C) and drained. 4 ml of guanidium HCl, pH 5.2, 25 mM sodium citrate, was added, and the suspension was harvested by scraping and transferred to sterile 15-ml polypropylene tubes (Falcon). The RNA was sheared by passing the mixture through a 21-gauge needle 10 times. N-Lauryl sarcosine was then added to 0.5% and absolute ethanol was added to 50%. The tubes were placed at −20 °C overnight and then centrifuged at 7500 × g at 4 °C for 15 min to pellet the RNA. The RNA pellet was resuspended in 800 μl of guanidium HCl, transferred to a microcentrifuge tube, and 400 μl of cold (−20 °C) absolute ethanol was added. The tubes were placed at −20 °C for 4 h, and the RNA recovered by centrifugation in a microcentrifuge for 15 min. The pellet was washed twice in 70% ethanol, resuspended in 200-μl of proteinase-K buffer, and treated with proteinase-K (200 ng/ml) for 30 min at 37 °C (22). 660 μl of guanidium HCl and 400 μl of cold
Results

Synergistic Cytotoxicity of TNF and Cycloheximide on TA1 Cells—We had noted in earlier experiments that cells treated concurrently with TNF and cycloheximide die within a few hours. To determine the time required for this cytotoxicity, TA1 cells were plated in 96-well microtiter plates and treated with TNF alone or in combination with cycloheximide for 0.5–6.0 h. The results of this experiment on preadipocytes and adipocytes is shown in Fig. 1, A and B, respectively. The results indicate that virtually complete lysis of TA1 preadipocytes and adipocytes occurs by 4–6 h after incubation with both agents together, and appreciable cytotoxicity was observed within 1–2 h of treatment. TNF alone is not toxic to these cells, and cycloheximide, at the concentration used, exhibits only 20–50% reduction in cell number over the 6-h pulse, which is mainly due to an inhibition in cell proliferation compared with the control cells rather than cytotoxicity. Even after 24 h of treatment with cycloheximide, the survival for the TA1, TA1 R-6, and mouse L-929 cells is 36, 38, and 40% of control, respectively.

Selection of Resistant Variants and Characterization of Resistant Clone R-6—Based on the above results, we developed a strategy for isolating variants resistant to the combined action of these agents. TNF (0, 0.1, 1.0, 10, and 100 ng/ml) and cycloheximide (0, 1, 10, and 100 μg/ml) were titered to determine the optimal concentrations for TNF-mediated killing of TA1 cells in the presence of cycloheximide. The optimal concentration of cycloheximide was 10 μg/ml. At this dose, the cytotoxicity due to cycloheximide alone was minimal, and the synergy with TNF yielded cytotoxicity sufficient to select rare, resistant variants. TNF-induced cytotoxicity could be readily detected at 0.1 ng/ml and appeared to plateau at 10–100 ng/ml (see Fig. 2A). We therefore chose 10 ng/ml of TNF and 10 μg/ml of cycloheximide as appropriate concentrations for selection of TNF-resistant variants. 5 × 10⁶ cells were treated with TNF (10 ng/ml) and cycloheximide (10 μg/ml) for 6 h, then washed free of the selecting agents, and refed complete medium. No mutagenesis was used to select these variants. This schedule was repeated weekly for 3 weeks, and the surviving colonies were isolated by ring cloning, expanded, and analyzed for resistance to TNF and cycloheximide. The frequency of surviving cells from a series of these experiments was in the range of 1 × 10⁻⁷ to 1 × 10⁻⁶.

Among the several clones isolated, TA1 R-6 was chosen for detailed analysis of its response to TNF and cycloheximide because it was highly resistant to the cytotoxic effects of TNF and cycloheximide used together, but retained some TNF-mediated functions. Other mutants with different properties will be described elsewhere. The relative degree of resistance of the TA1 R-6 cell line was determined by treating both TA1......
Fig. 3. Morphology of cell lines. A, TA1 cells confluent, uninduced, and stained with crystal violet. B, TA1 cells induced with indomethacin (125 μM) and dexamethasone (1 μM) for 3 days and stained with oil red-O. C, confluent TA1-R6 stained with crystal violet. D, TA1-R6 treated with TNF (10 ng/ml) for 3 days, stained with crystal violet. E, TA1-R6 treated with 10 ng/ml TNF and 1 μM dexamethasone for 3 days, stained with crystal violet, and F, TA1-R6 treated with TNF 10 ng/ml and indomethacin (50 μM) for 3 days and stained with crystal violet. The magnification in A and B is greater than in C–F in order to show the accumulation of lipid droplets; lower magnification is more suitable for C–F to demonstrate focus formation and the effects of TNF on the TA1-R6 cells.

R-6 and the parental TA1 cells with cycloheximide and various concentrations of TNF for 8 h. The results, shown in Fig. 2A, indicate that in the presence of cycloheximide, as little as 0.1 ng/ml of TNF is required for nearly complete cytotoxicity of the parental cell line, while 100 ng/ml of TNF did not yield significant toxicity in the TA1 R-6 cell line, a greater than 1000-fold increase in resistance. To further characterize the response of these cells, cytotoxicity induced by TNF was determined at two concentrations of cycloheximide. Fig. 2B indicates that an 8-h treatment of these cells with either 10 or 100 μg/ml of cycloheximide results in approximately 20 and 60% cytotoxicity, respectively. The cytotoxicity is not appreciably increased by increasing concentrations of TNF. Since, the cytotoxicity of cycloheximide at 10 μg/ml at
24 h is not appreciably different for the parental TA1, the TA1 R-6, or TNF-sensitive mouse L-929 cells, we conclude that the relative resistance of TA1 R-6 cells to TNF/cycloheximide treatment is a consequence of an altered response to TNF, not cycloheximide.

Morphology and mRNA Expression of Mutant and Parental Cell Lines—Morphologically, the mutant TA1 R-6 cell line appears more transformed than the TA1 parental line. Fig. 3A shows the normal morphology of the undifferentiated TA1 cells. These cells become contact inhibited, form a uniform monolayer, and exhibit well-defined nuclei and cytoplasm. Treatment of these cells with dexamethasone and indomethacin for 3 days induces them to differentiate into adipocytes with the accumulation of large lipid droplets in the cytoplasm (19, 20). These droplets appear as the dark staining material in the perinuclear region of these cells when stained with oil red-O (Fig. 3B). The TA1 R-6 mutant cells, in contrast, grow to much higher densities (1 x 10⁷ cells/10-cm plate versus 1 x 10⁶ cells/10-cm plate for the TA1 cells), are not contact inhibited, and tend to pile on each other and form foci (Fig. 3C).

The TA1 R-6 cells cannot morphologically differentiate into adipocytes even following treatment with known inducers of differentiation such as indomethacin or dexamethasone. To analyze the capability of the TA1, TA1 R-6, and mouse L-929 cell lines to induce adipocyte specific mRNAs, the cells were treated with indomethacin (125 μM) and dexamethasone (1 μM). RNA was harvested at 3 days after treatment and analyzed for expression of two adipocyte-specific mRNAs, AP2 and clone 47. The results, shown in Fig. 4, indicate that TA1 cells demonstrate marked induction of these adipocyte specific mRNAs, while the TA1 R-6 and mouse L-929 cell lines exhibit no detectable expression of these adipocyte specific mRNAs, even after treatment with indomethacin and dexamethasone for 3 days.

Response of TA1 and TA1 R-6 Cells to TNF—TNF causes an inhibition of adipocyte-specific gene expression in TA1 adipocytes and reverts the phenotype of these cells to the fibroblast-like state. Furthermore, treatment of TA1 preadipocytes with TNF can block the conversion of preadipocytes to adipocytes (13). In our attempts to determine whether TNF would exert any effect upon the phenotype of the TNF-resistant TA1 R-6 cell line (see above), we noted that while the TA1 R-6 cell line is fully resistant to the cytoxic effects of TNF and cycloheximide in an 8-h assay, it exhibits a marked sensitivity to the cytoxic effects of TNF when used alone for 2-4 days. Fig. 3D shows the appearance of TA1 R-6 cells after a 3-day exposure to TNF at 10 ng/ml. Only residual cellular debris, which stains darkly with crystal violet but lacks distinct nuclear and cytoplasmic structures, is observed. Viability was determined as a function of time of exposure to 10 ng/ml of TNF. The results, shown in Fig. 5, indicate that cytoxicity is evident by 2 days and is maximal at about 4 days of exposure in TA1 R-6 cells. This behavior is characteristic of the prototypical TNF-sensitive mouse L-929 cells in which cytoxicity requires 2 days to become evident and 3-4 days to become maximal. The dramatic alteration in TNF sensitivity of this cell line relative to the parental TA1 cell line may be related to its apparent "transformed" growth behavior.

Inhibition of TNF Cytotoxicity with Dexamethasone and Indomethacin—Based on the knowledge that TNF is generally cytoxic to transformed but not normal cells, and the fact that, in TA1 cells, dexamethasone and indomethacin induce the differentiated state (19, 20), tests were performed to determine whether these differentiation-inducing agents altered the susceptibility of the parental or mutant TA1 cells to the actions of TNF. The mutant TA1 R-6 line was particularly interesting in this respect because of its transformed morphology and its unexpected cytoxic response to TNF.

From the experiments shown in Fig. 1, A and B, we know that both preadipocytes and differentiated TA1 cells are rapidly killed by the combined action of TNF and cycloheximide. The time course for TNF and cycloheximide toxicity is slightly delayed in adipose cells compared to preadipose cells, but this difference is minimal. There is, therefore, little evidence that the state of differentiation appreciably alters the sensitivity of TA1 cells to the combination of TNF and cycloheximide. TA1 R-6 cells were treated with either dexamethasone (1 μM) or indomethacin (125 μM) concurrently with TNF for 3 days to determine whether either of these differentiation-inducing drugs could alter the cytoxicity of TNF. These results, shown in Fig. 3, E and F, indicate that dexamethasone and indomethacin can block the cytoxic effects of TNF on these cells. Interestingly, the cells treated with indomethacin and TNF develop a unique spindle-shaped morphology that is not observed in the parental cell line (F). This morphology is not observed in TA1 R-6 cells treated with indomethacin alone (data not shown) and is not observed in cells treated with dexamethasone and TNF (E). This suggests that an interaction between these two agents is responsible for the altered morphology. However, this altered phenotype is not required for resistance to TNF-mediated cytoxicity since dexamethasone, which on its own does not affect morphology, still blocks cytoxicity. To further verify the anti-cytoxic effects of dexamethasone on these cells, a time course of treatment of TNF in the presence or absence of dexamethasone was performed (Fig. 5). These results indicate that dexamethasone can completely block the cytoxic effects of...
TNF on these cells over the 4-day period of exposure to TNF.

Since the TA1 R-6 cell line cannot establish a fully differentiated state, as measured by its inability to accumulate adipocyte-specific mRNA (Fig. 4), we surmise that these agents are not inhibiting TNF cytotoxicity by inducing the differentiated state. It is more likely that these drugs interact directly or indirectly with one or more of the pathways by which TNF elicits its toxic effects. To assess these possibilities, the concentration of dexamethasone and indomethacin required to inhibit TNF cytotoxicity were determined in mouse L-929 cells, which are sensitive to the cytotoxic effects of TNF, but do not differentiate to adipocytes. The results indicate that both dexamethasone (Fig. 6A) and indomethacin (Fig. 6B) block the cytotoxic effects of TNF in mouse L-929 cells. Since mouse L-929 cells do not develop a differentiated adipocyte phenotype upon treatment with indomethacin or dexamethasone, one must conclude that the induction of a specific differentiated state is not required for suppression of the cytotoxic effects of TNF. Moreover, the mechanism by which these drugs block TNF cytotoxicity is not exclusive to the TA1 R-6 mutant or cells of the adipocyte lineage.

The concentration range over which dexamethasone blocks TNF cytotoxicity is broad, with 50% inhibition requiring about 10–20 nM of dexamethasone (Fig. 6A). Indomethacin, in contrast, is effective over only a narrow range of concentrations, (30–200 μM) (Fig. 6B), since above 300 μM indomethacin is itself toxic. The effective concentration range for indomethacin is two to three orders of magnitude above that required to inhibit cyclooxygenase (prostaglandin synthetase), but overlaps the concentration range that is optimal for induction of the differentiated, adipocyte phenotype (20). This may indicate that the biochemical processes by which indomethacin induces differentiation are similar, if not identical, to those involved in blocking TNF cytotoxicity. Finally, dexamethasone and indomethacin may be blocking the cytotoxicity of TNF at different points in the pathway or by different mechanisms. Dexamethasone, while very potent at inhibiting the cytotoxic effects of TNF, only modestly stimulates TA1 cell differentiation, whereas indomethacin at 30–200 μM markedly accelerates the conversion of preadipocytes to adipocytes (Fig. 4) and Ref. 20.

**TNF Versus TNF Plus Cycloheximide Cytotoxicity—**To further distinguish whether TNF cytotoxicity of TA1 cells operates through similar biochemical pathways in both the killing induced by TNF (alone) in 3–4 days or the accelerated (3–6 h) killing of TNF in the presence of cycloheximide, TA1 cells exposed to TNF and cycloheximide for 6 h were pretreated with indomethacin or dexamethasone. If the biochemical mechanisms by which TNF causes cytotoxicity are the same as those mechanisms by which TNF and cycloheximide cause cytotoxicity, we might expect dexamethasone or indomethacin to also inhibit toxicity caused by TNF and cycloheximide. However, indomethacin and dexamethasone, given concurrently with TNF/cycloheximide, did not provide measurable protection against cytotoxicity. Moreover, pretreatment of the TA1 cells with either indomethacin or dexamethasone for 0.5, 1, 3, 7, or 21 h prior to TNF/cycloheximide exposure did not provide measurable protection against cytotoxicity even if indomethacin and dexamethasone were kept in the media during the period of TNF/cycloheximide exposure (Table I). These results demonstrate that neither indomethacin nor dexamethasone reverse the toxicity of TNF and cycloheximide, regardless of the duration of drug exposure, and provide further evidence that the mechanism by which TNF induces rapid cytotoxicity in the presence of cycloheximide is different from the mechanism by which it induces protracted cytotoxicity in sensitive cells.

**DISCUSSION**

TNF has a multitude of activities each of which could be mediated through a variety of complex signal-transduction mechanisms. In an effort to begin to distinguish and elucidate...
these pathways, variants were selected that were resistant to
the synergistic cytotoxicity of TNF and cycloheximide. These
variants were made in adipogenic TA1 cells in which TNF
has been shown to inhibit and reverse differentiation (13).
Individual variants might allow the analysis of common and
independent pathways by which TNF induces cytotoxicity or
inhibits differentiation.

Preadipocytes and differentiation were analyzed to deter-
mine whether the state of differentiation altered the sus-
ceptibility of these cells to TNF plus cycloheximide. Toxicity is
evident after only 1–2 h of exposure and causes virtually
complete lysis of these cells by 4–6 h, with only a slight
difference in the time required for killing preadipocytes com-
pared to adipocytes. Mouse L-929 cells are also susceptible to
this combination, but they require 8–10 h for complete lysis
of the cells. Apart from the differences in the time course of
killing, the cytotoxic effect of TNF and cycloheximide in
combination does not appear to be specific for cell type or
state of differentiation. The mechanism of killing is not
known but apparently has no requirement for ongoing protein
synthesis and is effective on a short time scale. These facts
may be most simply accounted for by the accumulation of a
toxic metabolite of a pathway induced by TNF. Such a met-
abolite may be a normal product of a TNF-induced signal-
transduction pathway that is not metabolized due to the block
in protein synthesis. This could in turn imply that a critical,
but labile, enzyme may be involved in preventing the accu-
mulation of a toxic metabolite.

The potent synergism between TNF and cycloheximide was
utilized to select rare, resistant variants by repeated 6-h pulses
of TNF and cycloheximide once a week over a 3-week period.
One variant, TA1 R-6, was isolated that is completely resist-
ant to the cytotoxicity induced by TNF in the presence of
cycloheximide but is sensitive to TNF alone. This variant has
a more transformed appearance, grows to higher densities, is
not contact inhibited, and tends to form foci. Furthermore,
this cell line does not morphologically differentiate into an
adipocyte and is unable to express adipocyte-specific mRNAs,
even following treatment with indomethacin and dexameth-
asone. Interestingly, the TA1 parental cell line only expresses
differentiated functions when the cells have reached conflu-
ence and have become growth arrested (19, 20). The failure of
TA1 R-6 cells to become contact inhibited may be related to
their failure to express adipocyte-specific RNAs and
undergo differentiation to adipocytes. Alternatively, the ge-
netic change that leads to the transformed phenotype may be
responsible for the inability of TA1 R-6 cells to differentiate
since introduction of known oncogenes into adipogenic cells
prevents their differentiation (24).

Many transformed cell lines are susceptible to cytotoxicity
by TNF alone whereas normal cells are not. This cytotoxicity
is different from the toxicity induced in the presence of
cycloheximide since it requires 2–4 days instead of 2–4 h.
While the TA1 R-6 cell line is completely resistant to the
combined cytotoxicity of TNF and cycloheximide, it is ex-
tremely sensitive to the cytotoxicity that occurs with TNF
alone. In the parental TA1 cell line, TNF can block the
conversion of preadipocytes to adipocytes and is not toxic to
them during a 2–4-day exposure. Since transformed cells are
susceptible to the cytotoxic effects of TNF alone and normal
cells are not, it is possible that the changes that caused the
TA1 R-6 cell line to develop a more transformed phenotype
also caused susceptibility to TNF toxicity. Since these cells
are fully resistant to the toxicity induced by TNF and cyclo-
heximide in 2–4 h, but are extremely sensitive to the cytotoxic-
ity induced by TNF alone in 2–4 days, the mechanisms by
which TNF induces toxicity in each case is likely to be independent.

Since tumor cells, but not normal cells, are susceptible to
the cytotoxicity induced by TNF, it was of interest to deter-
mine whether the state of differentiation altered the cytotoxic
effects of TNF, either alone or in combination with cyclo-
heximide. To test this, mouse L-929 cells were analyzed. Mouse
L-929 cells have no adipogenic potential, even when treated
with indomethacin and dexamethasone, but are susceptible
to TNF. Remarkably, indomethacin and dexamethasone,
given concurrently with TNF, are capable of blocking the
toxic effects of TNF in these cells. These results indicate the
following two things: 1) the ability of these drugs to block
TNF cytotoxicity is independent of the capacity of the cells
to develop a specific differentiated state, and 2) the mecha-
anism for protection by these drugs is not a phenomenon
restricted to the TA1 R-6 cell line.

Dose-response analysis of these drugs in TA1 R-6 and
mouse L-929 cells indicates that dexamethasone is active half-
maximally at approximately 10 nM; this is consistent with the
protective effect against TNF cytotoxicity being mediated by
the glucocorticoid receptor (25). Moreover, dexamethasone
can block TNF-mediated repression of ferritin mRNA expres-
sion,2 and it is possible that dexamethasone may act to block
the induction or repression of other genes regulated by TNF,
genes that may be critical for inducing cytotoxicity. Interest-
ingly, indomethacin is functional only in the range of 30–200
μM. Since complete inhibition of cyclooxygenase occurs with
indomethacin at concentrations of 1 μM, these results would
indicate that inhibition of cyclooxygenase (and, therefore,
prostaglandin production) is not sufficient to block the cyto-
toxic effects of TNF. We have previously reported that induc-
tion of differentiation of TA1 preadipocytes to adipocytes by
indomethacin is optimal at 30–200 μM (11). It, therefore,
seems plausible that activation of pathways involved in the
differentiation of these cells is also involved in the protection
of these cells from the cytotoxic effects of TNF and that the
mechanisms by which indomethacin and dexamethasone
block TNF action are distinct from one another.

Kettlehut et al. (26) recently described the prevention of in
vivo toxic effects of TNF by cyclooxygenase inhibitors. Rats

2 T. R. Reid and G. M. Ringold, unpublished observations.
Two Mechanisms for TNF Cytotoxicity; however, no data that is currently available addresses this issue.

We have isolated a mutant cell line, TA1 R-6, which is resistant to the cytotoxicity of TNF in the presence of protein synthesis inhibition. The TA1 R-6 cells are not defective in the TNF receptor since TNF is still capable of inducing a normal cytotoxic response in these cells. However, these cells are defective in those processes that lead to cytotoxicity in the presence of cycloheximide and TNF, and in addition, fail to express the adipocyte phenotype or characteristic adipocyte mRNAs. The results of these studies suggest that TNF activates at least two distinct or overlapping signaling pathways that can be functionally dissociated pharmacologically and genetically.

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