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Neutrophils recovered from inflammatory exudates possess increased levels of IL-8, but exposure of neutrophils to chemoattractants results in only a modest stimulation of IL-8 generation. This study was undertaken to explore the hypothesis that IL-8 generation in these cells is dependent upon the process of migration. Neutrophils synthesized up to 30 times as much IL-8 during migration in response to a gradient of diverse chemoattractants than they did when stimulated directly by the attractants in the absence of a gradient. This IL-8 response was dependent on migration since it was not observed in cells exposed to concentration gradients of chemoattractants under conditions that prevented cell movement. While actinomycin-D (1 μg/ml) had little influence on the generation of IL-8 during chemotaxis, the protein synthesis inhibitor cycloheximide (10 μg/ml) markedly blunted the accumulation of cell-associated IL-8, suggesting that new protein synthesis from preexisting mRNA was responsible for the effect. Consistent with this interpretation, migrating cells incorporated over 10 times as much [3H]leucine into IL-8 as did nonmotile neutrophils exposed to chemoattractants. A substantial portion of the IL-8 generated during chemotaxis was released upon subsequent metabolic stimulation. Thus, the IL-8 synthesized during chemotaxis is uniquely positioned to exert a regulatory influence on inflammatory processes governed by neutrophilic leukocytes responding to inflammatory and infectious stimuli. *The Journal of Immunology, 1999, 162: 1077–1083.*

Neutrophils activated by various metabolic agonists, including opsonized zymosan, PMA, LPS, granulocyte-macrophage-CSF, and TNF-α, synthesize and release several cytokines including TNF, IL-1, IL-6, IL-8, and IL-12 (1–6). These factors are important regulators of the inflammatory response. In particular, IL-8 is a potent chemoattractant that promotes the mobilization of neutrophils into sites of infection and inflammation. Recent studies have demonstrated that, in some instances, protein synthesis rather than release of stored material accounts for the enhanced secretion of IL-8 by activated neutrophils (7). Activated neutrophils potentially modulate IL-8 synthesis at either the level of mRNA transcription or translation. Northern blot analysis revealed increased expression of IL-8 mRNA upon stimulation of neutrophils with certain cytokines (8, 9), LPS (9, 10), culture supernatants (11), and lectin ligands (12). Transcriptional regulation of IL-8 has also been shown in several other systems, including epithelial cells infected with Helicobacter pylori (13) or Pseudomonas aeruginosa (14) and monocytes responding to cytokines (15). In addition to transcription, IL-8 generation during inflammation may also be regulated at the level of mRNA translation. Kuhns and Gallin found that IL-8 production in ionophore-stimulated neutrophils was partially suppressed by cycloheximide, a protein synthesis inhibitor (7). Under similar conditions, the RNA synthesis inhibitor actinomycin-D had little effect, suggesting that the synthesis of IL-8 was under translational rather than transcriptional control.

After migration to sites of infection and inflammation, neutrophils putatively release enhanced amounts of IL-8 and other cytokines in response to metabolic stimulation. At these sites, cytokines secreted by chemotactic neutrophils exert potent effects on many aspects of the inflammatory response (16–18). However, the ability of chemotactically responsive cells to release IL-8 upon further stimulation has not, to our knowledge, been directly tested. In preliminary experiments, we observed that neutrophils recovered after chemotactic migration not only displayed a remarkably enhanced ability to release IL-8 upon metabolic stimulation but also possessed markedly elevated levels of IL-8 in comparison with nonmotile cells that were exposed directly to chemoattractants, suggesting that IL-8 synthesis may have occurred during chemotactic migration. The present investigation was undertaken to explore this possibility. The results indicate that chemotactic migration induces IL-8 synthesis by activating a cellular process that results in enhanced translation of mRNA present in resting cells.

Materials and Methods

Human IL-8, ELISA IL-8 assay kits, and monoclonal anti-IL-8 Abs were obtained from R&D systems (Minneapolis, MN). Platelet-activating factor (PAF) was obtained from Calbiochem (San Diego, CA). Tissue solubilizer NSC-II was from Amersham Canada (London, Ontario, Canada). Precast gels and reagents for SDS gel electrophoresis were from Bio-Rad (Hercules, CA). Endothelial cell culture reagents were from Cell Systems (Kirkland, WA). Tissue A/G plus beads were from Oncogene Science (Cambridge, MA). Dulbecco’s minimal essential media (DMEM) was from Grand Island Biological (Grand Island, MD). Zymosan, FMLP, recombinant C5a, PMA, casein, DMSO, HBSS, cytocholin-D, actinomycin-D, and cycloheximide were obtained from Sigma (St. Louis, MO). Cycloheximide was dissolved in distilled water at a concentration of 1 μg/ml.

Abbreviations used in this paper: PAF, platelet-activating factor; DMEM, Dulbecco’s minimal essential media; ZAS, zymosan-activated serum.
glutamine, 20% colostrum-free bovine serum, 20% phils were washed and suspended at a final concentration of 1.0 erythrocytes were removed by isotonic ammonium chloride lysis. Neutrophil responses during transendothelial migration, an endothelial tractant at approximately the same rate. In experiments designed to exam-
ments revealed that the smaller pore size did not markedly restrict the diffusion of macromolecules from lower to upper compartments of chemotaxis chambers, as assessed with blue dextran (Fig. 1). While the pore size of these filters is not small enough to impede the transfer of even large molecules, the pore density of the 0.4-μm filters is markedly greater than that of the 3.0-μm filters, as assessed microscopically. Thus, cells in the upper compartments of chambers with either 3.0- or 0.4-μm pore size polycarbonate filters at the base of inserts of 24-mm Transwell chambers (Costar, Cambridge, MA) at an average density of 1×10⁴ cells/well, as previously described (21). The inserts were returned to wells containing 0.5 ml of endothelial cell growth media and cultured until confluent growth was attained (3–5 days), at which time the inserts were rinsed and used for chemotaxis assays as described below.

**Chemotaxis assay**

Chemotaxis was performed using 24-mm Transwell chambers (Costar), wherein the chemotaxtractant was separated from cells within replaceable inserts by either an untreated or endothelialized 3-μm pore size polycarbonate membrane. To expose neutrophils to chemotaxtractant gradients under conditions that physically limited migration, a 0.4-μm pore size filter, through which no migration was observed, was used. Preliminary experiments revealed that the smaller pore size did not markedly restrict the diffusion of macromolecules from lower to upper compartments of chemotaxis chambers, as assessed with blue dextran (Fig. 1). While the pore size of these filters is not small enough to impede the transfer of even large molecules, the pore density of the 0.4-μm filters is markedly greater than that of the 3.0-μm filters, as assessed microscopically. Thus, cells in the upper compartments of chambers with either 3.0- or 0.4-μm filters were exposed to a continuously increasing concentration gradient of chemotaxtractant at approximately the same rate. In experiments designed to examine neutrophil responses during transendothelial migration, an endothelial monolayer was grown on the filters as described above. Chemotaxtractants were deposited in lower compartments in a final volume of 1.5 ml and prewarmed to 37°C. After warming, 1×10⁷ neutrophils in 1.0 ml of HBSS were pipetted onto the detachable inserts, which were placed over the chemotaxtractant solutions. Loaded chambers were incubated for 90 min at 37°C in a humidified atmosphere of 5% CO₂ in air. At the end of incubation, the cells that migrated into the bottom chambers were dislodged by gentle scraping, harvested, and enumerated electronically. In some experiments, cells and media from upper chambers were recovered after incubation and processed for IL-8 analysis. Suspensions of recovered cells were cooled on melting ice and pelleted by centrifugation at 500×g for 5 min. Supernatants were recovered and saved for assay of IL-8 levels.
These chemoattractants but did not secrete detectable levels of IL-8. Cells that migrated in response to other chemoattractants to these chemoattractants released a small portion of this increased levels of IL-8. Cells that migrated in response to other chemoattractants in the absence of a gradient. Cells migrating in response to other chemoattractants (PAF, FMLP, C5a) also possessed levels of IL-8 that were markedly greater than those of cells that were directly stimulated with these chemoattractants but did not secrete detectable levels of IL-8 into the extracellular medium during chemotaxis. Cells retrieved from upper chambers after migration did not possess similarly increased levels of IL-8 (not shown).

**Results**

**Effect of chemoattractants on IL-8 synthesis**

Several chemoattractants including ZAS, PAF, FMLP, C5a, and casein were characterized for their ability to induce IL-8 synthesis in neutrophils. With the exception of PAF, each of these chemoattractants induced a potent chemotactic response resulting in the migration of 44–53% of upper chamber cells into the lower compartments. The response induced by PAF, which attracted 24% of upper chamber cells, was lower than that induced by other chemoattractants but still markedly greater than that observed in chambers without chemoattractant, wherein less than 1% of the cells deposited in the upper chambers were recovered in lower compartments (Table I).

When added to neutrophils in the absence of a chemotactic gradient, each of the chemoattractants used induced a moderate increase in cell-associated IL-8; levels of released IL-8 remained undetectable (Table I). The chemoattractants that induced the strongest migratory responses, ZAS and casein, induced the highest increase in cell-associated IL-8, increasing constitutive levels over 10-fold. With other chemoattractants, migratory responses, and IL-8 synthesis did not appear to correlate. Thus, PAF, FMLP, and C5a each induced a similar increase in IL-8 levels over that found in unstimulated cells but varied considerably in chemotactic potency (Table I). Lower levels of chemoattractants (1:10, 1:100, and 1:1000 dilutions of levels of ZAS, C5a, and FMLP reported in Table I) did not result in marked increase in the IL-8 response (not shown).

Neutrophils recovered after migration to each of the chemoattractants were found to possess far greater levels of IL-8 than levels observed in cells exposed directly to chemoattractants. ZAS and casein were again the most potent attractants in this respect; levels of IL-8 in cells responding to these attractants were over 100 times greater than levels found in unstimulated cells and 10- to 30-fold higher than levels found in cells stimulated with chemoattractants in the absence of a gradient. Cells migrating in response to these chemoattractants released a small portion of this increased IL-8. Cells that migrated in response to other chemoattractants (PAF, FMLP, C5a) also possessed levels of IL-8 that were markedly greater than those of cells that were directly stimulated with these chemoattractants but did not secrete detectable levels of IL-8 into the extracellular medium during chemotaxis.

**Table I. Levels of IL-8 in chemotactic- and chemoattractant-treated neutrophils**

<table>
<thead>
<tr>
<th>Chemoattractant</th>
<th>Chemotaxis&lt;sup&gt;a&lt;/sup&gt; (cells × 10&lt;sup&gt;5&lt;/sup&gt;)</th>
<th>Chemotactically-stimulated cells&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Chemotactic cells&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell associated (pg/10&lt;sup&gt;6&lt;/sup&gt; cells)</td>
<td>Released</td>
<td>Cell associated (pg/10&lt;sup&gt;6&lt;/sup&gt; cells)</td>
</tr>
<tr>
<td>None</td>
<td>&lt;0.1</td>
<td>51 ± 7.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>ZAS</td>
<td>4.55 ± 1.1</td>
<td>648 ± 40</td>
<td>ND</td>
</tr>
<tr>
<td>PAF (5 nM)</td>
<td>2.40 ± 0.7</td>
<td>143 ± 18</td>
<td>ND</td>
</tr>
<tr>
<td>FMLP (10 nM)</td>
<td>4.40 ± 1.1</td>
<td>120 ± 12</td>
<td>ND</td>
</tr>
<tr>
<td>C5a (2.9 × 10&lt;sup&gt;−8&lt;/sup&gt; M)</td>
<td>4.50 ± 0.9</td>
<td>160 ± 15</td>
<td>ND</td>
</tr>
<tr>
<td>Casein (0.5 mg/ml)</td>
<td>5.30 ± 0.9</td>
<td>560 ± 32</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> The number of responding cells was determined by electronic particle analysis of an aliquot of lower chamber fluid.<br>
<sup>b</sup> Neutrophils were stimulated by direct addition of chemoattractants in the absence of a chemoattractant gradient for 90 min at 37°C.<br>
<sup>c</sup> Cells were recovered after chemotactic migration through 3-μm filters. Released activity reflects levels found in lower compartment fluids after removal of cells by centrifugation and corrected for cell concentration. Cell-associated levels reflect levels recovered in washed cells.<br>
<sup>d</sup> Value represents IL-8 levels observed in resting cells (mean and SD of three experiments).<br>
<sup>e</sup> ND, not detectable (<10 pg/10<sup>6</sup> cells).

**Involvement of migration in the IL-8 response**

The results of the experiments described above indicate that exposure of neutrophils to gradients of chemoattractants potentiates IL-8 synthesis to an extent that is not observed in cells exposed to attractants in the absence of a gradient. While exposure of cells to chemoattractant gradients may directly induce IL-8 synthesis, the response may be secondary to processes activated during migration induced by these gradients. Experiments were designed to explore the actual role of chemotactic migration in induction of the IL-8 response. In the first approach, we employed a system to expose cells to a gradient of chemoattractant under conditions that prevented migration by limiting the pore size of the membrane within chemotaxis chambers to 0.4 μm (see Fig. 1). As shown in Table II, cells exposed to chemoattractant gradients produced higher levels of IL-8 than did cells directly exposed to chemoattractants, whether or not migration was allowed to proceed. However, cells recovered after migration displayed markedly higher levels of IL-8 than did cells exposed to chemoattractant gradients under conditions in which migration was prevented.

In a second approach, we examined the influence of the actin polymerization inhibitor cytochalasin-D (22) on chemoattractant-induced IL-8 synthesis. Preliminary experiments demonstrated

**Table II. Requirement of migration for the chemoattractant-induced IL-8 response**

<table>
<thead>
<tr>
<th>Treatment Conditions&lt;sup&gt;a&lt;/sup&gt;</th>
<th>IL-8 Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell associated (pg/10&lt;sup&gt;6&lt;/sup&gt; cells)</td>
</tr>
<tr>
<td>No gradient</td>
<td>436 ± 76</td>
</tr>
<tr>
<td>Gradient/permmissive conditions</td>
<td>4327 ± 357</td>
</tr>
<tr>
<td>Gradient/migration physically limited</td>
<td>1881 ± 97</td>
</tr>
<tr>
<td>Gradient/migration biochemically limited</td>
<td>706 ± 95</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cells were exposed either to a single concentration of chemoattractant (10% ZAS) in the absence of a gradient or to a concentration gradient of chemoattractants under either permissive conditions or conditions that physically or biochemically limited migration. Physical limitation of migration was effected by employing a 0.4-μm pore size filter in chemotaxis chambers. Biochemical inhibition was effected by addition of the actin polymerization inhibitor cytochalasin-D (10 μg/ml). IL-8 analyses were carried out using cells recovered from lower chambers when permissive conditions were used and from upper chamber under restrictive conditions.

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To demonstrate the de novo synthesis of IL-8 during chemotactic migration, we examined cells after migration through chemotaxis chambers. In the presence of cytochalasin-D, IL-8 levels induced by chemotactic gradients were similar to those induced in neutrophils by direct exposure to chemoattractants in the absence of gradients. These results are consistent with the possibility that the IL-8 response in neutrophils exposed to chemotactic gradients depends, in part, upon actual cellular migration.

**Effect of RNA and protein synthesis inhibitors on the chemotaxis-dependent IL-8 response**

The studies described above demonstrate that chemotactic neutrophils possess enhanced quantities of IL-8 as a result of processes activated during migration. However, the origin of this IL-8 is not clear. To investigate the origin of chemotaxis-induced IL-8, we examined IL-8 levels of cells induced to migrate in the presence of the protein and RNA synthesis inhibitors cycloheximide and actinomycin-D, respectively. Actinomycin-D (1 μg/ml) had little effect on IL-8 increases associated with chemotactic migration (Fig. 2). In contrast, cycloheximide (10 μg/ml) markedly attenuated increases in both cell-associated and released IL-8 during chemotactic migration. These observations are consistent with the proposal that increases in IL-8 levels observed in migrating neutrophils result from increased protein synthesis directed by pre-existing mRNA.

**IL-8 synthesis in migrating neutrophils**

To demonstrate the de novo synthesis of IL-8 during chemotactic migration, we employed neutrophils preincubated with L-[4,5-3H]leucine as described under Materials and Methods. The results shown in Fig. 3 demonstrate that chemotactic migration resulted in over a 10-fold increase in [3H]leucine incorporation into IL-8 as compared with cells treated with chemoattractant directly. Furthermore, when cells were treated with cycloheximide (10 μg/ml), the incorporation of L-[4,5-3H]leucine into IL-8 during migration was reduced by 40%.

**IL-8 synthesis by neutrophils during transendothelial migration**

To evaluate chemotaxis-dependent IL-8 synthesis in a physiologically relevant system, we examined cells after migration through endothelial monolayers. The results demonstrated that migration of neutrophils through endothelial monolayer results in markedly potentiated increases in cell-associated IL-8. In these experiments, neutrophils that migrated through endothelial monolayers were found to possess 6989 ± 1250 pg of IL-8/10^6 cells. During migration, these cells released 2050 ± 125 pg of IL-8/10^6 cells into the surrounding medium. Exposure of neutrophils to an endothelial cell monolayer in the absence of chemoattractants did not result in elevated levels of neutrophil IL-8. The amount of IL-8 released by endothelial cells cultured on inserts and exposed to 10% ZAS in either the absence or the presence of neutrophils (1.0 × 10^6 cells in 1.0 ml) was below the limits of detection of the assay used. Thus, the elevated IL-8 levels observed in neutrophils migrating through the endothelial monolayer could not be attributed to uptake of IL-8 released from stimulated endothelial cells.

**Release of IL-8 by metabolic stimulation of chemotactic neutrophils**

Finally, we examined the ability of neutrophils to release the IL-8 that was generated during chemotaxis in response to metabolic stimulation. Cells were recovered from the lower compartment of chemotaxis chambers after migration induced by 10% ZAS, washed, and exposed to 100 nM PMA for 30 min at 37°C. Results were compared with those obtained with neutrophils from the same preparation that were not subject to chemotactic migration or chemoattractant exposure. Upon exposure to PMA, chemotactic cells released 3132 ± 156 pg IL-8/10^6 cells while control cells

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**Table III. Effect of cytochalasin-D on chemoattractant-stimulated IL-8 generation**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ZAS</th>
<th>FMLP</th>
<th>C5a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell associated</td>
<td>Released</td>
<td>Cell associated</td>
</tr>
<tr>
<td>Untreated</td>
<td>281 ± 8</td>
<td>140 ± 8</td>
<td>62 ± 18</td>
</tr>
<tr>
<td>Cytochalasin-D</td>
<td>324 ± 6</td>
<td>130 ± 10</td>
<td>174 ± 12</td>
</tr>
</tbody>
</table>

* Untreated or cytochalasin-D (10 μg/ml)-treated neutrophils were stimulated by direct addition of chemoattractants for 10 min at 37°C and assayed for IL-8 generation as described in the text. Values (pg/10^6 cells) reflect levels above those observed in unstimulated cells (mean ± SD of three determinations). Chemoattractant concentrations were the same as those used for the experiments described in Table I.
neutrophils were characterized for their metabolic reactivity after migration but rather as a result of cellular process activated by migration.

Although cellular migration apparently played a major role in inducing IL-8 synthesis, we cannot entirely discount the possibility that migration facilitated a response that was triggered by a more traditional mechanism. Many cells, including neutrophils, are encumbered by an “adaptation” response that continuously resets the magnitude of responses to certain stimuli, including chemotactants (see Ref. 28 and references therein). In some noteworthy instances, the rate at which the cell resists is such that no response is initiated by small rates of stimulus increase; at higher rates of stimulus increase, the cell will respond maximally. Our data (Fig. 1) demonstrate that nonmigrating neutrophils in upper compartments of chemotaxis chambers are exposed to a linear increase in chemotactant levels. It is possible that such a linear increase is not sufficient to trigger an IL-8 response. However, when the cells are migrating through the filter toward the chemotactant, the rate of increase of attractant to which they are exposed would increase appreciably, probably in an exponential manner. Such an exponential rate of increase may be sufficient to trigger the maximal IL-8 response. If this is the explanation for the results observed, it should be possible to initiate with chemotactants a maximal response in static cells by ramping the stimulus concentration exponentially. Experiments are underway to determine whether this interesting hypothesis provides the basis for our dramatic results.
While heterogeneity in neutrophil responses to chemoattractants may have influenced our results, we do not believe neutrophil subpopulations can explain the dramatic increase in IL-8 levels observed in cells that have migrated. Indeed, there are well-documented precedents for heterogeneous responses of neutrophil subpopulations to chemoattractants and other metabolic stimuli (30–33). Subpopulation heterogeneity results in differences in a diverse array of responses, including Ca\(^{2+}\) mobilization, surface Ag expression, phagocytosis, and surface electric charge. There may be differences in migratory ability as well. Thus, it is conceivable that cells selected on the basis of migration are enriched in a subpopulation that displays a very brisk IL-8 response to chemoattractants, independent of cell migration. However, it should be noted that, under the conditions used, a high percentage of upper chamber cells migrated in response to each chemoattractant. Such a large subpopulation cannot account for the discrepant IL-8 values between chemoattractant-stimulated and chemotactic cells, since this population would markedly elevate levels in the initial cell preparations as well. Small subpopulations of cells that migrate and exhibit exceptionally elevated responses to chemoattractants independent of migration may play a role in the responses observed, but it is difficult to envision how such an effect explains the dramatic results shown in this study. However, many factors may influence the final tally of average IL-8 levels within such a complex population of cells. It may be necessary to evaluate IL-8 levels within individual cells to finally resolve the influence of cell migration on IL-8 generation in neutrophilic leukocytes.

The nature of the processes activated during migration that lead to enhanced IL-8 levels remains to be defined, but our results provide some important insights regarding the mechanisms involved. Thus, while the protein synthesis inhibitor cycloheximide markedly attenuated the response, the RNA synthesis inhibitor actinomycin-D had little effect. In addition, it is clear that de novo synthesis of IL-8 contributed to the response, since radiolabeled amino acids were incorporated into the parent molecule during migration. Therefore, chemotaxis apparently potentiates the translation of preexisting mRNA. Previous studies have demonstrated regulation of neutrophil IL-8 synthesis at both the levels of RNA transcription and translation. For example, Kuhns and Gallin observed inhibition of ionophore-stimulated IL-8 by cycloheximide but not actinomycin-D (7), similar to the effects found herein. In contrast, using Northern blot analyses, Cassatella et al. demonstrated increased levels of IL-8 mRNA in neutrophils stimulated with LPS (10). In addition, Hachicha et al. recently reported increased levels of IL-8 mRNA in neutrophils phagocytosing zymosan and certain microbial pathogens (18). The failure of actinomycin-D to inhibit responses in the present study cannot be taken as absolute evidence for the conclusion that transcription was not involved, since we did not directly validate the effect of the inhibitor on RNA synthesis. Thus, while actinomycin-D at levels known to interrupt mRNA synthesis in other systems had little influence on the IL-8 response reported in the present study, we cannot entirely exclude the possibility that increased expression of IL-8 mRNA may have contributed to this response.

IL-8 is a chemotactic cytokine that possesses the ability to attract cells to sites of inflammation and infection. IL-8 released by stimulated neutrophils is thought to exert important autocrine effects, amplifying the inflammatory response by recruiting additional cells to the affected area (34). The present study reveals an additional facet of this system of inflammatory regulation: the potentiation of IL-8 synthesis by chemotactic migration. How the IL-8 associated with neutrophils that arrive at inflammatory targets exerts its effect is unclear. However, cell-associated IL-8 would be released from cells that are either metabolically stimulated or disrupted after chemotaxis. In addition, cells with enhanced levels of IL-8 may possess heightened functional reactivity as a result of endogenous cytokine or in response to low but continuous levels of released cytokine. Finally, intracellular IL-8 potentially serves a paracrine-signaling function when intact but apoptotic neutrophils are ingested by macrophages.

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