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The Effects of Cytokines on Suppression of Lymphocyte Proliferation by Dexamethasone

Tom J. Creed,*† Richard W. Lee,* Paul V. Newcomb,* Alexandra J. di Mambro,*† Madhuri Raju,* and Colin M. Dayan2*†

Treatment failure occurs in up to 30% of patients treated with steroids for inflammatory diseases. The aim of this study was to explore the potential role of 21 cytokines in steroid-resistant inflammatory disease and to develop methods to restore steroid sensitivity through cytokine manipulation. The dexamethasone inhibition of lymphocyte proliferation assay correlates with the outcome of steroid therapy in ulcerative colitis (UC) and other inflammatory diseases. Using this assay, PBMC production of 21 cytokines, assayed by cytokine bead array, was correlated with percentage of suppression of proliferation by 10⁻⁶ M dexamethasone (Imax) in 26 healthy volunteers. Effects of the addition of exogenous cytokines to induce steroid resistance in PBMCs from healthy volunteers and cytokine blockade to improve steroid sensitivity in PBMCs from patients with steroid-resistant UC were then explored. Production of IL-1α, IL-10, IL-17, IFN-γ, G-CSF, GM-CSF, TNF-α, and IFN-inducible protein 10 (IP-10) correlated significantly with in vitro steroid sensitivity; however, only IL-2 and TNF-α reduced steroid sensitivity when added exogenously. Addition of IL-10 enhanced steroid suppression. Immunoneutralization or receptor blockade of IL-2, but not TNF-α, IFN-γ, IL-4, IL-17, or IP-10 increased steroid sensitivity in cells from steroid-resistant UC patients. Neutralization of IL-10 reduced steroid sensitivity. Of the large panel of cytokines studied, IL-2 appears to have the greatest antagonistic effect on the antiproliferative effect of steroids. These data suggest that IL-2 inhibition in vivo may improve the response to steroids in steroid-resistant individuals. The Journal of Immunology, 2009, 183: 164–171.

Glucocorticoids are widely used as immunosuppressive agents to treat inflammatory conditions. They have the advantage over other potent immunosuppressants of rarely producing profound immunosuppression, but the disadvantage of causing multiple dose-related side effects such as glucose intolerance, sleep disturbance, osteoporosis, hypertension, and centripetal obesity. In addition, it is a recurrent observation that up to 30% of patients with inflammatory disease fail to respond adequately to steroids. This failure to respond is not adequately explained by greater disease severity, and the frequency of steroid nonresponders is relatively consistent across a wide range of inflammatory conditions including ulcerative colitis (UC), asthma, and rheumatoid arthritis (1–4).

We have previously provided evidence that failure to respond to steroids in UC correlates with failure of PHA-stimulated peripheral blood lymphocytes to be suppressed by dexamethasone in an in vitro assay (4), and others have made similar observations in other diseases (1, 5–7). In vitro steroid resistance measured in this way varies widely across the normal population with 20–30% of individuals showing marked resistance. Furthermore, the degree of resistance in a given individual remains relatively constant over time, at least for healthy individuals (8), although the presence of inflammation may exacerbate the problem in the diseased state (9–13). Taken together, these in vitro and in vivo observations have lead us to hypothesize that steroid resistance is at least in part an inherent property of an individual irrespective of the presence of an inflammatory disease, which becomes relevant (and clinically apparent) in individuals who develop a disease that requires steroid therapy. Of note, no correlation was seen between in vitro lymphocyte steroid sensitivity and sensitivity to steroids in other tissues unrelated to the immune system (8, 14), suggesting that factors beyond the glucocorticoid receptor specific to individual tissues are responsible for steroid sensitivity. No consistent differences in glucocorticoid receptor (GR) affinity or number have been observed that are sufficient to account for the variation (2, 8, 15, 16).

Data from the early 1990s suggested that exposure to cytokines can induce steroid resistance in lymphocytes in vitro, notably IL-2 and IL-4 (12). The combination of IL-2 and IL-4 reduces GR ligand-affinity binding in T cells in vitro (12) by a mechanism that involves p38 MAPK activation (10). Increased expression of IL-2 and IL-4 mRNA has been reported in airway cells from bronchial-veolar lavage in steroid-resistant asthmatics (17), although these transcripts did not feature in a recent differential expression microarray study comparing alveolar macrophages in steroid-sensitive and steroid-resistant patients (9). Both IL-2 and IL-4 in combination were required to induce steroid resistance in these initial studies. However, where T cells have been studied in systems that also involve TCR-mediated activation (e.g., by Ag or anti-CD3), IL-2 alone has been shown to induce steroid resistance in vitro both in murine and human cells (18, 19). We have recently shown...
that blockade of IL-2 signaling with the anti-CD25 mAb basiliximab can increase steroid sensitivity in PHA-stimulated PBMCs in vitro and subsequently generated pilot data for the in vivo use of basiliximab in combination with steroids in patients with steroid-resistant UC (20, 21).

The role of a number of other cytokines in steroid resistance has also been studied. Exposure to IL-13 has been shown to reduce GR affinity and promote steroid resistance, although this effect seems to be on monocytes rather than T cells (11) and IL-15 exposure to IL-13 has also been shown to reduce steroid resistance in monocytes and T cells in vitro and subsequently generated pilot data for the in vivo use of basiliximab in combination with steroids in patients with steroid-resistant UC (20, 21).

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Steroid resistance in inflammatory bowel disease has been variously defined (28, 29). In this study, steroid-resistant patients with a diagnosis of UC were defined as: 1) patients who had had a colectomy for ongoing disease activity despite corticosteroid treatment, 2) patients who had demonstrated at least one episode of active colitis with an Ulcerative Colitis Symptom Score ≥6 (30), despite at least 2 wk of prednisolone ≥30 mg/day, or 3) patients who had demonstrated at least one episode of Truelove and Witts measures of disease severity (28), with poor indicators of outcome (31), despite at least 3 days of i.v. steroids.

### Cytokines and Abs

The following cytokines and neutralizing anti-cytokine Abs were obtained from R&D Systems: IL-4 (204-IL/cf), IL-10 (217-IL/cf), IL-17 (317-IL/cf), TNF-α (210-ta/cf), IFN-γ (285-IF/cf), IP-10 (266-IP), anti-human TNF-α (MAB610), anti-human IL-2 (neutralizing) (MAB202), anti-human IL-10 (MAB217), anti-human IP-10 (MAB266), anti-human IFN-γ (MAB285), anti-human IL-17 (MAB317), and mouse IgG1 isotype control (MAB002). IL-2 was purchased from BD Biosciences (reference 554603).

Other Abs and drugs licensed for human therapeutic use were obtained as follows: basiliximab, a chimeric human/mouse mAb against...
the high-affinity \( \alpha \)-chain of the IL-2R, CD25 (Novartis Pharmaceuticals), daclizumab, a humanized anti-CD25 mAb (Roche Pharmaceuticals), and infliximab, a humanized monoclonal anti-TNF-\( \alpha \) Ab (Schering-Plough Pharmaceuticals).

**In vitro measurement of steroid sensitivity (Imax)**

The sensitivity of peripheral blood T lymphocytes was assessed in a functional assay as previously described (1, 4, 8). Briefly, \( 4 \times 10^6 \) PBMCs isolated by gradient density centrifugation were incubated in triplicate with PHA at optimized final concentrations of 5–20 \( \mu \)g/ml in the presence or absence of a suprapharmacological concentration of dexamethasone (\( 10^{-6} \) M). Cell proliferation was assessed by tritiated thymidine incorporation after 48 h. Results were calculated as cpm from the mean of triplicate cultures and presented as percentage inhibition at 10\( \times 10^5 \) cells/ml. Imax was assessed by tritiated thymidine incorporation after 48 h. Results were calculated as cpm from the mean of triplicate cultures and presented as percentage inhibition at 10\( \times 10^5 \) cells/ml.

**Measurement of cytokine production**

PBMCs were stimulated with PHA for 48 h in microtiter plates as outlined above. One hundred sixty-five microliters of supernatant was removed from the wells and commercially assayed for 21 cytokines simultaneously by Luminex Research using Luminex cytokine multiplex bead array technology (32). This technique employs a system of latex microbeads dyed with fluorophores to create an array of simultaneous immunomicroarray. The lower limit of detection for all cytokines was 3.2 pg/ml.

**Addition of cytokines to proliferating lymphocyte cultures in healthy volunteers**

To determine the potential antagonist effect on steroid sensitivity of raising cytokine levels, selected, recombinant cytokines were added to the culture period of the Imax assay in the dose range 2.5–100,000 pg/ml.

**Blockade of cytokines in proliferating lymphocyte cultures in patients with steroid-resistant UC**

To determine the potential of cytokine antagonism to improve steroid sensitivity, anti-cytokine/cytokine receptor Abs were added to the lymphocyte cultures. Concentrations of control murine IgG1 over the same range were used to correct for nonspecific effects. Results were calculated and corrected for nonspecific inhibition from the addition of Ig as follows: Change in Imax = (Imax with cytokine blockade + dexamethasone) – (Imax with addition of IgG1 control + dexamethasone).

**Statistics**

Correlation of cytokine supernatant concentration with Imax was undertaken using Spearman’s \( \rho \) coefficient as the cytokine level data were not normally distributed. Differences in Imax between healthy volunteers and patients with steroid-resistant UC were analyzed with the Mann-Whitney \( U \) test. Change in Imax in response to cytokine inhibition or cytokine blockade was analyzed by ANOVA.

**Results**

**Characteristics and steroid sensitivity of subjects**

Table I shows characteristics of the 12 patients with steroid-resistant UC. All of the patients had inactive disease at the time of blood sampling and all were studied a minimum of 1 year after administration of any biological agent derived drugs. Patients with steroid-resistant UC (\( n = 12 \)) had significantly less inhibition of lymphocyte proliferation (median suppression = 56.1%) in the presence of a supramaximal dexamethasone dose (\( 10^{-6} \) M) compared with healthy individuals (\( n = 37 \)).

**Time course of cytokine release in response to PHA**

The time course of cytokine accumulation of six cytokines (IL-2, IL-4, IL-5, IL-10, TNF-\( \alpha \), and IFN-\( \gamma \)) in the supernatant of PBMC cultures from healthy volunteers was studied by multiplex bead
array analysis (supplemental Fig. 14). Although TNF-α was released earlier than the other cytokines, peak cytokine levels were seen at the 48-h time point for all six cytokines and this time point for cytokine sampling was therefore used in subsequent assays.

**Correlation between steroid resistance and cytokine production by stimulated lymphocytes**

To determine whether variation in cytokine production by lymphocytes between individuals is a possible determinant of steroid resistance by an autocrine mechanism, 48-h PHA-stimulated cytokine levels were measured in isolated peripheral lymphocytes from 26 healthy volunteers, and the results were correlated with Imax levels in the same individuals (Table II). Production of IL-1α, IL-10, IL-17, G-CSF, GM-CSF, IP-10, IFN-γ, and TNF-α correlated negatively with Imax (p < 0.05, r > 0.4). Interestingly, production of IL-2 and IL-4 was low in these cultures and failed to correlate with Imax despite the fact that both of these have been previously reported to play a role in steroid resistance (9, 10, 12, 18, 33).

**The effect of cytokines upon steroid sensitivity of PBMC from healthy volunteers**

Recombinant cytokines were added exogenously to PHA-stimulated T lymphocyte cultures in the presence of high-dose dexamethasone (10⁻⁶ M) to determine their effect upon Imax (Fig. 2). IFN-γ, IP-10, IL-10, and IL-17 had no significant effect over the dose ranges studied (100–100,000 pg/ml). The effect of IL-4 addition reached significance by ANOVA, but no consistent dose-response pattern was seen (Fig. 2). TNF-α significantly reduced steroid sensitivity in the dose range 10,000–100,000 pg/ml. IL-2 also reduced steroid sensitivity at a concentration of 2500 pg/ml (Fig. 2).

**The effect of cytokine blockade on steroid sensitivity in steroid-resistant patients with UC**

Neutralizing Abs to selected cytokines were used to inhibit individual cytokine action. In addition, the effects of three anti-cytokine Abs used in clinical practice were also studied as potential modifiers of steroid resistance. Neutralizing Abs were used at 0.1, 1, 10, and 100 times (anti-TNF) or 1, 10, and 100 times (anti-IL-2, anti-IL-4, anti-IL10, anti-IL-17, anti- IFN-γ, anti-IP-10, anti-IL-10, anti-IL-4, anti-IL-17, anti- IFN-γ, anti-IP-10, anti-IL-10).
>95% neutralizing concentrations; manufacturer’s data), or 0.1, 1, 10, and 100 times therapeutic serum levels (basiliximab, daclizumab, and infliximab).

Fig. 3 shows the effects of cytokine blockade on the proliferation of PHA-stimulated lymphocytes from steroid-resistant patients in the presence of $10^{-6}$ M dexamethasone. Results are shown as percentage increases in Imax (more steroid sensitive).

Changes in Imax due to IgG alone were subtracted from Imax changes seen with specific Abs to give the final corrected Imax change attributable to specific cytokine blockade.

Neutralization of IL-10 decreased Imax (reduced steroid sensitivity; Fig. 3), consistent with removal of the immunosuppressive effects of this cytokine. Neutralization of IL-4, IL-17, IP-10, TNF-$\alpha$, or IFN-$\gamma$ had no consistent effect (Fig. 3). Blockade of

FIGURE 3. The effect of exogenously added blocking Abs upon Imax in PHA-stimulated PBMC cultures in the presence of high-dose dexamethasone ($10^{-6}$ M) in patients with steroid-resistant UC ($n = 6$). Values of $p$ refer to ANOVA across all concentrations. Bars, Mean values with SEs.

FIGURE 4. Response of individual subjects to (a) the addition of three cytokines, IL-2, TNF, and IL-10 ($n = 9$), and (b) their blockade ($n = 12; n = 6$ for anti-IL-2 and anti-IL-10). Values are shown for the highest concentration of cytokine or Ab used in Figs. 2 and 3. The nonspecific effects of addition of IgG are shown (i). For cytokine blockade, comparison with the effects of the equivalent dose of IgG are shown.
IL-2 with neutralizing Abs or the monoclonal anti-CD25 Abs basiliximab and daclizumab resulted in a significant increase in steroid sensitivity (Fig. 3).

Fig. 4 shows the response of individual healthy volunteers to the highest doses of the three cytokines that had significant effects (Fig. 4a) and the response of individual steroid-resistant patients to blockade of these cytokines (Fig. 4b). It can be seen that irrespective of initial starting value, the Imax of all subjects falls with the highest dose of IL-2 and Imax falls in almost all subjects with TNF-α (Fig. 4a). By contrast, the response to addition of IL-10 is more varied, adding up to the minimal overall effect seen in Fig. 2. Note, however, that only 3 of 11 healthy volunteers had a starting Imax of <80%, and hence we have limited data on whether such “steroid-resistant healthy volunteers” do behave differently from the steroid-sensitive individuals. Fig. 4b shows the broad spread of Imax values among the steroid-resistant patients studied (with 8 of 11 having initial Imax values <80%) and the marked non-specific effects of the highest dose (10 μg/ml) of control IgG (Fig. 4bi). In addition, the very consistent effects of IL-2 blockade acting to increase steroid sensitivity (Fig. 4b, iii and iv) and of IL-10 blockade in reducing steroid sensitivity (Fig. 4bii) can be seen and contrast with the inconsistent effects of TNF blockade (Fig. 4, v and vi).

Discussion

Following T cell stimulation with PHA, we have observed a correlation between cytokine secretion levels and steroid resistance in vitro for 8 of 21 cytokines studied (IL-1α, G-CSF, GM-CSF, IP-10, IFN-γ, TNF-α, IL-10, and IL-17), 5 of which were selected for further study because of their known or potential effects on T cells along with IL-2 and IL-4 which have previously been described as influencing steroid sensitivity. However, for four of these when studied individually (IP-10, IFN-γ, IL-4, and IL-17), we did not observe evidence of a causative role in steroid resistance as addition of exogenous cytokine or neutralization of endogenous production had no consistent effect on steroid sensitivity (Figs. 2 and 3).

Addition of IL-2 and TNF-α reduced steroid sensitivity. The doses required were 10- to 100-fold higher than the levels detected in the supernatants of stimulated cells (Fig. 2 and Table II). However, it is likely that for autocrine effects, the concentration of cytokine immediately surrounding secreting cells is considerably higher than that in the supernatant as a whole. At the concentrations studied, IL-2 appeared more potent at inducing steroid resistance, increasing Imax by a median of 29.8% (95% confidence interval, 11.9–47.8%) at a concentration of 2500 pg/ml, compared with a median increase of 14.3% (95% confidence interval, 0.3–21%) with 100,000 pg/ml TNF-α (Fig. 2). In contrast to TNF-α, we failed to observe a correlation between levels of IL-2 production and Imax in healthy volunteers (Table II). However, if levels are 100–200 times higher in the immediate cellular milieu, in part due to immediate IL-2 consumption by IL-2R-expressing cells (34), these data are consistent with a role for either or both cytokines in the variation in steroid resistance between individuals.

To further address this question of whether endogenous production of cytokine was at levels sufficient to influence steroid resistance, we explored the effect of immunoneutralization of cytokines or receptor blockade on steroid sensitivity. It was necessary to perform these experiments in our cohort of steroid-resistant individuals (Fig. 1), as in steroid-sensitive subjects the degree of suppression by steroids is frequently so high that any additional effect of cytokine blockade would not be apparent (Fig. 4a). Both neutralization of IL-2 or blockade of its receptor in steroid-resistant individuals enhanced steroid sensitivity significantly as we have previously observed (20). However, somewhat surprisingly, neutralization of TNF-α had no effect on steroid sensitivity (Fig. 3), with the individual data showing wide variation in effects between subjects (Fig. 4b, v and vi). Neutralization of IL-10 reduced steroid sensitivity, consistent with the removal of an immunosuppressive cytokine. Fig. 4 confirms that where significant effects were seen in the overall data these were reflected in consistent effects across all or almost all individuals. Taken together, these data suggest, at least in the context of the PHA-stimulated cultures examined, cellular IL-2 production significantly contributes to steroid resistance, while IL-10 has the opposite effect. Although TNF-α has the potential to antagonize steroid action following T cell activation (Fig. 3), as previously reported in monocytes (23), the results of the inhibition experiments suggest that, under the culture conditions studied, the levels of TNF-α reached are not sufficient to contribute significantly to steroid resistance in the majority of subjects. TNF-α inhibition alone, not necessarily used concomitantly with steroids, has also been shown in vivo to induce remissions in UC and asthma (13, 24), providing evidence that TNF plays a role in these disease processes, although not necessarily by increasing steroid resistance. IFN-γ has been reported to promote glucocorticoid signaling in resting T cells exposed to IL-2 and IL-4 (9), but did not appear to have any effect as a single agent on activated T cells in the current system. For IL-10, although increased IL-10 production was associated with reduced steroid sensitivity (negative correlation coefficient, Table II), exogenous IL-10 augmented the response to steroids (Fig. 2) and anti-IL-10 reduced it (Figs. 3 and 4), consistent with a previous report on asthma (26).

The exact mechanism by which IL-2 promotes steroid resistance remains unclear but several possible pathways have been described. A reduction in steroid bioavailability mediated through the induction of the multidrug-resistant receptor family may contribute to steroid resistance. The MDR-1 gene product is P-glycoprotein 170 which actively transports steroids and other drugs out of the cell and has been reported to be up-regulated by IL-2 (35). Additional mechanisms included an alteration in the ligand-binding affinity of the GR (10, 12) via p38 MAPK activation (described in monocytes (10)), inhibition of GR translocation by complex formation with STAT5 (36), and IL-2-induced retention of the transcription factor FOXO3 required for glucocorticoid action in the cytoplasm (37). In addition, in T cells activated by anti-CD3, IL-2 (and anti-CD28) amplifies the level of activation, resulting in increased c-fos expression (via ERK activation) and hence higher levels of AP-1 which may prevent nuclear translocation of GR (18). We have recently reported that cells expressing an intermediate level of IL-2R (CD25) before activation are more steroid resistant than CD25-negative cells (38) and these findings are additionally consistent with a important role for IL-2 in modulating steroid resistance.

Healthy volunteers were used for the studies of cytokine production and the effects of exogenous cytokines because these individuals can be studied free of immunosuppressive drugs and we have previously provided evidence that steroid resistance is an inherent property of individuals irrespective of the presence of disease (4, 8). For studies of cytokine blockade to enhance steroid sensitivity, a very steroid-resistant population of subjects was required (as it is not possible to measure the increase in steroid sensitivity in individuals who are already very steroid sensitive; see baseline levels in Fig. 4). For this we used patients with UC in remission who had previously behaved as steroid resistant and the availability of these subjects is a key strength of the current study. As can be seen from the patient demographics in Table I, some
patients were receiving concomitant medications, notably azathioprine. However, repeat analysis \((n = 4)\) excluding these individuals produced similar results for basiliximab \((p = 0.002)\), a similar trend for daclizumab \((p = 0.08)\), anti-IL-2 \((p = 0.09)\), and anti-IL-10 \((p = 0.1)\), and no effect for the remainder of the Abs as before \((p values 0.4–1.0)\). Potential limitations of our study are the use of a nonphysiologic stimulus (PHA) and that we have studied peripheral blood rather than specifically cells known to be involved with disease. We chose the use of PHA as a stimulus since we have shown in three separate series (one prospective \(4\)) and two retrospective (Ref. 20 and Fig. 1 of the current paper) that the maximal degree of suppression of PHA-induced proliferation in vitro correlates with the outcome of steroid treatment in vivo, and this has been replicated by others in other conditions \((1, 6)\). Furthermore, this data and the finding of “in vitro steroid resistance” in 20–30% of healthy volunteers \((8)\) suggest that steroid resistance is an inherent property of an individual’s circulating lymphocytes, not just a problem in the lymphocytes at the site of the disease involved in the autoimmune process. Hence, there is a high likelihood that conclusions drawn from the effects of manipulation of the cytokine exposure of cells in the dexamethasone suppression assay are relevant to the response to steroids in disease and, in the case of IL-2 blockade, we have preliminary evidence that this is true \((20, 21)\).

Note that our approach of initially identifying cytokines of potential relevance by relating levels of production with Inmax (Table II) may miss potent cytokines released in small quantities into the immediate milieu of cells, cytokines that are rapidly locally consumed or ones that only have effects on steroid resistance in combination with other cytokines. Goleva et al. \((9)\) have recently reported the results of differential expression mRNA array analysis comparing alveolar macrophages from steroid-sensitive and steroid-resistant asthma and reported increased levels of TNF, IL-1, IL-6, and IL-8 and a range of chemokines. However, it is still possible that this approach may miss subtle differences in cytokines with potent effects, and additional studies of candidate cytokines as well as specific combinations will be required to address these issues. Ideally, the data in Table II would have been analyzed by multiple regression to identify the relative potential contribution of different cytokines, but the nonparametric nature of this data precluded such an analysis. To address this deficiency in future studies, combinations of neutralizing Abs that have the potential to synergize effects should be explored.

Taken together, the observations described in this report indicate that of the cytokines studied, IL-2 appears to be the most potent inducer of steroid resistance, although as combinations of cytokines were not studied, it cannot be concluded that IL-2 has an exclusive role. Our data are also insufficient to determine whether variation in IL-2 production between individuals is an important determinant of the wide interindividual variation in steroid sensitivity since supernatant levels were low. However, our data do provide evidence that blockade of IL-2 action, in particular using the commercially available anti-CD25 Abs basiliximab and daclizumab, is more effective than any other cytokine inhibition studied at restoring steroid sensitivity in steroid-resistant individuals. This raises the possibility that blockade of this pathway may be used therapeutically to overcome steroid resistance and enhance the anti-inflammatory effects of steroids in vivo \((29)\). In support of the latter conclusion, we have recently reported that basiliximab was effective at inducing remission in steroid-resistant UC in an uncontrolled trial when used in combination with steroids \((20, 21)\). A recent study of IL-2 blockade in UC with daclizumab failed to show benefit \((39)\), but this may relate to the lower doses of concomitant steroid used.

In summary, we present the results of a wide-range study of the role of cytokines in steroid resistance and have provided evidence that IL-2 appears to represent a possible candidate to explain and treat steroid resistance. Inhibition of CD25 (IL-2R) is particularly attractive because CD25 expression is limited to lymphoid cells, hence general enhancement of the effects of steroids on nonlymphoid tissue, i.e., enhancement of unwanted steroid side effects, would not be expected to occur. Controlled clinical studies in steroid-resistant patients treated simultaneously with glucocorticoids are required to confirm both the efficacy and safety of this approach.

**Disclosures**

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