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REVIEW

The immunomodulatory actions of adenosine during systemic inflammation

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Abstract

The inflammatory response is elementary for the recognition and elimination of invading pathogens. However, during severe or persistent systemic inflammation, e.g. during sepsis or (auto)inflammatory diseases such as rheumatoid arthritis, the inflammatory response can also be detrimental to the patient. Finding ways to orchestrate the inflammatory response in a tailored fashion could be of great therapeutic value, i.e. by potentiating it when necessary to eliminate micro-organisms, and dampening the response in case of potential collateral tissue damage. In the last decades it has become increasingly clear that the signalling molecule adenosine exerts tissue-protective and immunomodulatory properties. Adenosine acts as an autocoid: the extracellular concentration of adenosine rapidly increases in situations of impending tissue danger, such as ischaemia and inflammation, and subsequent stimulation of membrane-bound adenosine receptor induces several effects, which can protect the affected tissue. Here, we discuss in detail how adenosine can modulate the immune response and how this action could potentially be exploited in the clinical arena in patients with inflammatory diseases such as sepsis.

Introduction

Already in 1983 Newby proposed the term 'retaliatory metabolite' to describe adenosine as a protective autocoid which can ameliorate tissue damage.[3] By binding to one of its four known receptors, designated A_1, A_{2a}, A_{2b} and A_3, adenosine is able to induce a variety of effects, such as vasodilation, ischaemic preconditioning, modulation of the sympathetic nervous system activity, and inhibition of inflammation. In concert, these effects have the potential to control cellular damage and to prevent further organ injury. Here we discuss how the adenosine system is influenced during inflammation, how adenosine subsequently modulates the immune response and how this action could potentially be exploited in patients with inflammatory diseases such as sepsis.

Adenosine

Adenosine is an endogenous purine nucleoside involved in a wide range of (patho)physiological processes. It was first described as an important signalling molecule by Drury and Szent-Györgyi in 1929,[4] demonstrating that adenosine reduces heart rate, lowers blood pressure and induces coronary vasodilatation. Approximately a decade ago, adenosine was recognised as a signalling molecule that is able to signal inflammation as well as modulate the inflammatory response.[5,6] During systemic inflammation, adenosine concentrations increase rapidly, thereby protecting the host from inflammation-induced tissue injury.

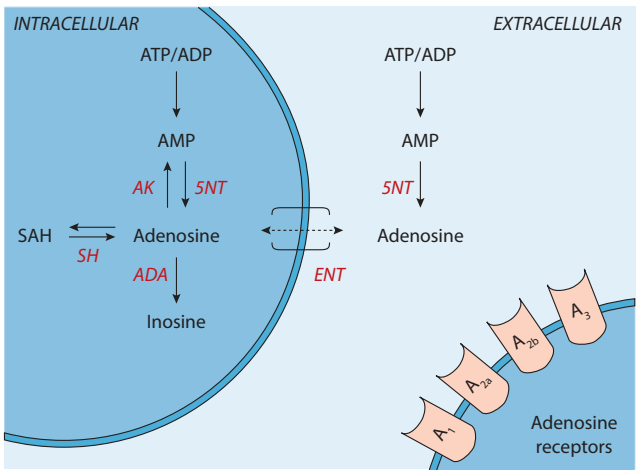
There are five recognised mechanisms by which adenosine is able to protect cells against inflammation-associated damage. First, adenosine directly decreases the pro-inflammatory response by binding to its specific receptors on different immune cells;[7] second, adenosine decreases the energy demand of cells via inhibition of parenchymal cell function, e.g. the negative inotropic effects on the heart muscle; third, adenosine improves the oxygen and nutrient availability by local vasodilatation;[4] fourth, adenosine preserves the endothelial barrier function by controlling local inflammation, promoting angiogenesis and neovascularisation;[8] and fifth, adenosine increases the intrinsic tolerance against ischaemia and reperfusion injury.[9]

Adenosine metabolism

Adenosine is formed both extracellularly and intracellularly by dephosphorylation of adenosine monophosphate (AMP) by 5-nucleotidase (both endo-5NT as well as ecto-5NT, the latter also referred to as CD73). 5NT is the rate limiting step in the breakdown of ATP,[10] and therefore acts as a crucial regulator

for the production of adenosine. 5NT is highly expressed in nearly all tissues, with the highest levels in the colon, kidney and brain.[11] Intracellular hydrolysis of S-adenosylhomocysteine (SAH) into adenosine and L-homocysteine also acts as a source of adenosine, albeit less important during pathophysiological conditions. Degradation of adenosine is mainly confined to the intracellular compartment, through adenosine deaminase (ADA) and adenosine kinase (AK). The equilibrative nucleoside transporter (ENT) controls facilitated diffusion between extracellular and intracellular adenosine. During well-oxygenated conditions, the intracellular concentration is lower than the extracellular concentration and adenosine is mainly transported into the intracellular compartment, where it is subsequently metabolised.[12] Adenosine metabolism is illustrated in *figure 1*.

Figure 1. Schematic illustration of the adenosine metabolism, adapted from Ramakers *et al*.[21] 5'-nucleotidase (5NT) catalyses the dephosphorylation of AMP, S-adenylhomocysteine hydrolase (SH) accounts for the hydrolysis of S-adenylhomocysteine (SAH); adenosine deaminase (ADA) and adenosine kinase (AK) are responsible for the degradation of adenosine, which mainly occurs intracellularly. The equilibrative nucleoside transporter (ENT) facilitates transmembranous adenosine transport.



Adenosine receptors and signalling

Adenosine elicits its effects by binding to one of its four known receptors, designated A_1, A_{2a}, A_{2b} and A_3. Under basal conditions, interstitial adenosine concentrations are very low (within the nanomolar range),[10] but sufficient to activate three of its receptors, namely, the A_1, A_{2a} and A_3 receptor.[13] The A_{2b} receptor has a lower affinity for adenosine (Km > 1 μM) and therefore requires higher concentrations of adenosine for activation.

Adenosine receptor signalling is complex. Not only does it vary between receptor subtypes, it also depends on the specific cell type involved.[14] Since adenosine receptors are present on all types of cells, its therapeutic potential differs between organ systems and diseases. Adenosine-related research mainly focuses on immune cells, such as macrophages, lymphocytes and dendritic cells, endothelial cells and cells of our central nervous system (e.g. cells in the cortex, cerebellum and hippocampus).[10]

Briefly, the A_1 and A_3 receptor are Gi/o protein coupled receptors. Activation of these receptors leads to a) the inhibition of adenylyl cyclase, which in turn decreases the 3',5'cyclic adenosine monophosphate (cAMP) concentration, and b) the activation of phospholipase C. Adenosine A_1 receptor stimulation is also linked to various kinase pathways, such as protein kinase C. In addition, A_1 receptor stimulation is also able to open K^+ channels and inhibit Ca^{2+} channels. Furthermore, adenosine A_3 receptor stimulation also utilises alternative pathways, such as Ras homologue gene family, member A (RhoA) and phosphatidylinositol 3 (PI3) kinase.[15]

The A_{2a} and A_{2b} receptors are Gs protein coupled receptors which, upon activation, result in increased cAMP concentrations. Subsequently, cAMP activates cAMP-dependent protein kinases. In general, the anti-inflammatory effects of adenosine A_{2a} receptor activation are mediated by a) the activation of protein kinases which interfere with the IκB kinase complex that selectively inhibits the NFκB pathway, b) the activation of cAMP-response element-binding (CREB) protein which mediates gene expression directly and indirectly by competing with the NFκB pathway and c) through the activation of the exchange factor activated by cAMP (EPAC).[15] In lipopolysaccharide (LPS)-stimulated macrophages, the expression of the A_{2a} and A_{2b} receptor is augmented whereas A_1 and A_3 receptor expression is attenuated. This increase in A_{2a} receptor number correlated with an increase in the potency of a specific A_{2a} receptor agonist to reduce tumour-necrosis factor alpha (TNF-α) release.[16] Also in humans, sepsis increases the expression of the adenosine A_{2a} receptor on circulating granulocytes. However, the receptor function is impaired, due to reduced ligand-binding affinity, thereby diminishing the anti-inflammatory potential of adenosine.[17]

Adenosine and inflammation

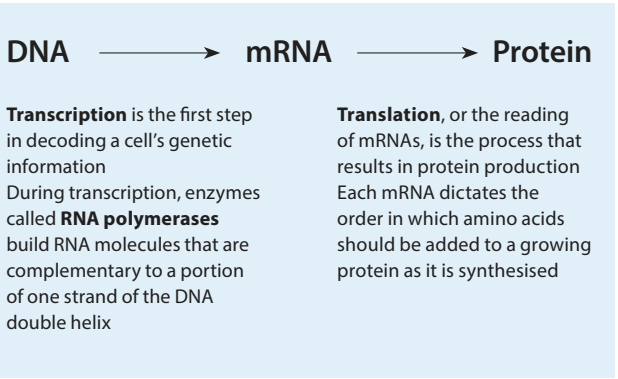
Under normal physiological conditions the adenosine concentration is within the nanomolar range, but during pathophysiological conditions, such as hypoxia or inflammation, its concentration increases rapidly up to tenfold during septic shock.[18]

How inflammation influences adenosine metabolism

Adenosine metabolism is able to change in circumstances associated with cellular damage. In order to describe inflammation induced changes in the adenosine metabolism in more detail, *figure 2* illustrates the process of transcription and translation. For hypoxaemia these changes have been studied in detail. During hypoxaemia, the adenosine concentration

increases rapidly as a consequence of changes in adenosine metabolism. In brief, the formation of adenosine is accelerated through increased activity of 5NT, which results in enhanced AMP breakdown/adenosine formation, whereas the cellular uptake and subsequent degradation of adenosine, by adenosine deaminase and kinase, are inhibited.[19] Moreover, the uptake of adenosine is delayed due to inhibition of the ENT further increasing its extracellular concentration.[19] Recently it was demonstrated that adenosine metabolism is also influenced by systemic inflammation. During murine peritonitis, the extracellular adenosine concentration increased rapidly as a consequence of increased formation of adenosine along with a decrease in degradation of adenosine.[20] Also in humans *in vivo*, systemic inflammation changes the circulating adenosine concentrations by altering its metabolism. In combination with a change in the expression of the various adenosine receptor subtypes, this promotes an anti-inflammatory and tissue protective response. More specifically, during experimental human endotoxaemia, the expression of 5NT mRNA was up-regulated, whereas ADA mRNA was down-regulated. Furthermore, the activity of both ADA and AK was significantly diminished. Interestingly, these changes in activity levels were not caused by an alteration in gene transcription. The changes in enzymatic activities of ADA, AK, and ecto-5NT were likely due to an effect on protein translation or a direct effect of inflammation on protein activity. The latter mechanism is most probable, given the findings that protein expression of ecto-5NT followed the change in its gene transcription.[21] *In vitro*, LPS dose-dependently attenuated the activity of both ADA and AK. Additionally, we demonstrated that the gene expression of the adenosine A_{2a} and A_{2b} receptor, which upon activation have potent anti-inflammatory effects,[22-25] increased. In contrast, the gene expression of the adenosine A_1 and A_3 receptor decreased. Taken together, the inflammation-induced changes in the metabolism of adenosine and adenosine receptor expression promote the anti-inflammatory potential of adenosine.

Figure 2. A schematic description of the process of transcription and translation. mRNA: messenger RNA.



How adenosine influences inflammation; the anti-inflammatory potential of adenosine

Previous *in vitro* studies have demonstrated that adenosine receptor stimulation, e.g. on peripheral blood mononuclear cells, attenuates the production of pro-inflammatory cytokines, such as TNF-α,[7] whereas it augments the release of the anti-inflammatory cytokine IL-10.[26] These findings have been confirmed in murine *in vivo* models, and are associated with increased survival during animal endotoxaemia.[27] However, it is important to realise that there is a pathophysiological difference between the consequences of immune modulation during endotoxaemia and caecal ligation and puncture (CLP)-induced sepsis. During endotoxaemia, only the inflammatory response itself is responsible for the observed tissue damage and mortality. Inhibition of the proinflammatory immune response will generally improve outcome. In contrast, during CLP-induced sepsis, the animals may develop organ dysfunction (eventually leading to death) due to the inflammatory response or bacterial dissemination. Because the proinflammatory response is necessary for the clearance of bacteria, but may also be detrimental during an overwhelming or persistent activation of the immune system, inhibition of the proinflammatory immune response can exert opposite effects in these models. In these experiments, outcome may improve in animals that die as a consequence of inflammation-induced collateral tissue damage, but may worsen in animals that die of uninhibited bacterial dissemination.

In humans *in vivo*, continuous intravenous administration of adenosine resulted in a significant attenuation of IL-6 production during experimental endotoxaemia.[28] However, the clinical applicability of administration of exogenous adenosine is limited due to its rapid cellular uptake and degradation[29] and important haemodynamic side effects when given in higher dosages. We have extensively reviewed the literature on the anti-inflammatory properties of adenosine elsewhere.[30]

The adenosine paradox

As already mentioned above, modulation of the immune response through interference with the adenosine system may exert both beneficial, but also detrimental effects. While adenosine receptor stimulation may limit an excessive inflammatory response and associated organ injury during systemic inflammation, inhibition of the immune response may exert deleterious effects during *active* infections. Interestingly, adenosine metabolism is not only relevant in the host, as bacteria have evolved and now also use the anti-inflammatory potential of adenosine to escape both the innate and adaptive defence mechanisms otherwise essential for its clearance.[31] Recent research shows that *Staphylococci* are able to enhance

adenosine formation through membrane bound adenosine synthase A which harbours two 5NT signature sequences. As such, *Staphylococci* exploit the anti-inflammatory potential of adenosine to attenuate the hosts pro-inflammatory response and thereby enhances bacterial survival. This clearly illustrates how attenuation of the pro-inflammatory response during active infection (with living bacteria) might be detrimental.

So, both adenosine receptor stimulation and inhibition should be addressed with caution, especially in patients with active infections. Nonetheless, even in sepsis patients, the anti-inflammatory response of adenosine may prove to be useful in situations during which antibiotics are started and bacterial clearance is no longer an issue. At that moment, down-regulation of the often persistent and hyperactive immune response, through adenosine receptor stimulation, might prevent further organ damage. When, for example, a patient is admitted to the ICU with sepsis/septic shock, adenosine-related therapy has a narrow window and could be of therapeutic value when the patient is still in the pro-inflammatory phase, but active infection is adequately treated with antibiotics.

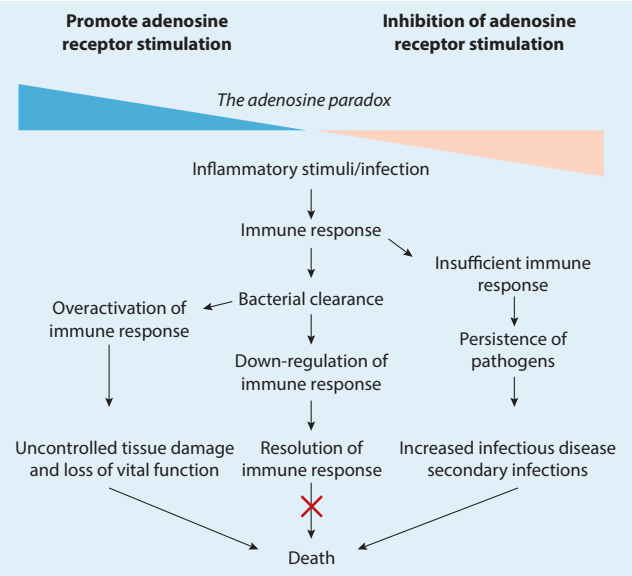
Furthermore, with the persistence of sepsis, patients often have reactivation of endogenous viruses and develop nosocomial infections with opportunistic pathogens. This is explained by a prolonged immunosuppressive state after a period of hyper-inflammation. In this 'secondary phase of immunoparalysis',[32] the patient could benefit from adenosine receptor inhibition to prevent further suppression of the immune response. Recently, Hasko and co-workers demonstrated that antagonism of the adenosine receptor improved survival in murine sepsis.[23] Thus, both stimulation and inhibition of the adenosine-receptor system may represent promising approaches to improve the outcome of sepsis patients, bearing in mind that timing of specific therapy is of paramount importance (*figure 3*).

Several immunomodulatory treatment modalities aimed at limiting the innate immune response during sepsis have not yielded positive results. Although most therapeutic targets in sepsis are based on concepts of pathogenesis, as previously excellently reviewed,[33] these negative study results might be explained by the fact that during sepsis, a plethora of complex interactions between the infecting microorganism and the host immune, inflammatory, and coagulation responses occur. Nonetheless, quite a few have proven very effective in the treatment of autoimmune diseases, such as rheumatoid arthritis.

Pharmacological approaches

Hitherto, pharmacological approaches aimed to mimic the role of adenosine have been evaluated in the field of cardiovascular and pulmonary medicine and autoimmune diseases. Numerous clinical trials using specific adenosine receptor agonists (mainly A_{2a} and A_3) as well as antagonists have been initiated

Figure 3. The adenosine paradox. During prolonged or over-activation of the immune system (left side of the figure) adenosine receptor stimulation will attenuate the innate immune response. During an insufficient immune response, inhibition of the adenosine receptor system may restore the immune response



in several patient groups, but none of them focus specifically on infection.[34]

In rheumatoid arthritis patients, adenosine A_{2a} and A_3 receptor expression is increased.[35] Moreover, the highest levels of A_{2a} and A_3 density are closely associated with the lowest levels of disease activity, suggesting that the endogenous activation of these receptors plays a role in the attenuation of disease activity. In a phase II trial, in patients with rheumatoid arthritis, treatment with increasing dosages of a specific A3 agonist (CF101) was able to improve clinical signs and symptoms of rheumatoid arthritis[36] associated with minimal adverse events; no relevant haemodynamic adverse effects were observed, except for headache. Possibly, specific A_{2a} receptor stimulation would also further attenuate disease activity.

Furthermore, the use of adenosine A_{2a} specific receptor agonists (compound CVT-3146) is studied in coronary imaging studies, using the A_{2a}-receptor-induced vasodilator effect to induce coronary vasodilatation.[37] However, the mechanism of action in these studies is vasodilation, a phenomenon which may be undesirable in patients with severe inflammation. To prevent unwanted cardiovascular effects, in particular hypotension, pharmacists have developed topical formulations such as dry powder for inhalation and vehicle gel. Several of these agents have been tested. In pulmonary medicine, compound UK-432097 reached phase II clinical trials as an agent for the treatment of chronic obstructive pulmonary disease (Safety and Efficacy of UK-432,097 In Chronic Obstructive Pulmonary Disease. NCT00430300). Sonedenoson (MRE0094) (2-[2-(4-

chlorophenyl)ethoxy] adenosine) entered phase II clinical trials for induction of healing of diabetic foot ulcers (Safety and Efficacy Study of MRE0094 to Treat Large, Single or Multiple, Chronic, Neuropathic, Diabetic Foot Ulcers, NCT00318214).

Alternative means of stimulation of the adenosine pathway have also been investigated in humans. Potential side effects of selective adenosine receptor agonists can be mitigated with the use of allosteric enhancers of the adenosine receptor. An allosteric enhancer selectively increases the efficacy of endogenous adenosine to stimulate receptors in tissues, thereby promoting adenosine receptor stimulation mainly at those times and in those tissues where the endogenous adenosine concentration is increased, avoiding potential systemic side effects of adenosine receptor agonists. At present, adenosine receptor allosteric modulators (e.g. T-62 for chronic pain and migraine headache) are in various stages of human clinical trials.

Besides the use of specific adenosine receptor agonists or antagonists (direct and allosteric), pharmacological interventions interfering with adenosine metabolism might have therapeutic potential. However, to optimise the effects of these pharmacological interventions, it is necessary to realise that adenosine metabolism itself changes during inflammation. Therefore, inflammation-induced changes in adenosine metabolism should be taken into account when targeting the different enzymes and transporters involved in adenosine metabolism.

It was recently demonstrated that blockade of 5NT either with the use of knockout mice or pharmacological blockade, and thus blockade of the formation of adenosine, increases sepsis-induced kidney and lung injury and eventually death.[38] Furthermore, recent studies demonstrate that 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors (statins) are able to activate ecto-5NT activity, thereby limiting myocardial ischaemia reperfusion injury.[39,40]

Our findings also suggest that the use of substances that target the intracellular metabolism will not have a substantial effect on the extracellular adenosine concentration, since the activity of adenosine-degrading enzymes is already reduced during inflammation.[21] Contrary, the use of dipyridamole, an adenosine reuptake inhibitor, augments the circulating adenosine concentration. Furthermore, during experimental endotoxaemia the dipyridamole-induced increase in the extracellular adenosine concentration was associated with a fierce increase of the anti-inflammatory cytokine IL-10.[41]

Also, the development of specific adenosine receptor agonists as pro-drugs might have therapeutic potential. These pro-drugs will only have an effect if the inactive form is synthesised.[42] Activation of such compounds, e.g. phosphorylated forms of agonists, require a dephosphorylation step to become active. Ecto-5NT, which is upregulated during inflammation, is such an enzyme and will therefore facilitate conversion of phosphorylated pro-drugs in the inflamed tissues. This approach may help to achieve the desired anti-inflammatory actions and decrease unwanted side effects, such as hypotension. To determine whether these new potentially therapeutic possibilities are feasible and effective in humans, *in vivo* studies are warranted.

Besides the precautions described in the different pharmacological approaches above, which focus mainly on hypotension as an unwanted side effect in the different treatment modalities, one should realise that there is a difference in intervening with the adenosine metabolism, and direct adenosine receptor binding. Our group previously studied the use of dipyridamole during human experimental endotoxaemia. We demonstrated that subjects pretreated with dipyridamole had significantly higher circulating adenosine concentrations, but we did not observe a significant change in haemodynamics.[41] Furthermore, in a study by Soop *et al.*, intravenous administration of adenosine did not result in haemodynamic changes during systemic inflammation.[28]

Do we need to start serving coffee at our ICU?

Caffeine, the world's most frequently used drug, is a nonselective adenosine receptor antagonist. Three of the adenosine receptors, A_1, A_{2a} and A_{2b}, form its major target. Already in the concentration range achieved by drinking two or three regular cups of coffee, significant blockade of the three mentioned adenosine receptors occurs.[13] Previous studies in animal models have shown that caffeine is able to potentiate the production of pro-inflammatory cytokines both *in vitro*[43] and *in vivo*[44] and exacerbate tissue injury during systemic inflammation.[45,46] Of interest, rats treated with caffeine showed improved survival during CLP-induced sepsis.[47] Whether enhanced bacterial clearance played a role in the observed improved survival is unclear. Given its potent pro-inflammatory effects, it would be worthwhile to investigate its effects during the immunosuppressive phase of sepsis when it might help restore the immune balance. However, the administration of caffeine during systemic inflammation induced by human

Human experimental endotoxaemia; 'The LPS model'

To study the innate immune response during systemic inflammation the standardised experimental human endotoxaemia model is frequently used. The administration of bacterial components in humans *in vivo* was first described in the 1890s when patients with cancer were treated with a mixture of killed bacteria aimed to reduce tumour growth. Since 1955, the LPS model has been extensively used for experimental purposes. The administration of purified *E. coli* LPS results in an acute systemic inflammatory response which enables to study the innate immune system in a controlled fashion. This model thereby provides the opportunity to study changes in inflammatory mediators besides haemodynamic, humoral and metabolic responses.[49]

experimental endotoxaemia, did not affect the inflammatory response nor the observed subclinical organ damage.[48]

Conclusions

To date, a vast amount of preclinical evidence indicates that adenosine receptor stimulation can control excessive inflammation. Although targeting of a single receptor has provided insight into the role of that specific receptor during inflammation, the direct effects of adenosine receptor stimulation or inhibition vary between receptor subtypes, cell types, and the different models of inflammation. It is also important to realise that adenosine is involved in a wide variety of different physiological systems through a complex signalling cascade, besides immunomodulation. Nonetheless, adenosine receptors remain an important molecular target for adenosine-based therapeutics, throughout the entire spectrum of inflammation. The success of adenosine-based therapies during situations of persistent, chronic or inappropriate inflammation, depend on a) the specific role of adenosine in the mechanism of disease and b) the timing of its actions with respect to the therapeutic window and the specific phase/progression of disease. Throughout the entire field of medicine, individualised, tailor-made therapies are introduced. Possibly, modulation of the inflammatory response, either by inhibition of the hyper-inflammatory state or stimulation of the inflammatory response during the phase of immune paralysis, with the use of the adenosine system, might be used in the treatment of patients, but more work is needed before it will find its way to the clinic.

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