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Pharmacogenomics of Response to Anti-Tumor Necrosis Factor Therapy in Patients with Crohn's Disease

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Contents

Abstract

The relatively recent development of genetically engineered agents has the potential to alter the treatment of Crohn's disease radically, and drugs that inhibit tumor necrosis factor-α (TNFα) have been introduced as a new therapeutic class with high efficacy, rapid onset of action, prolonged effect, and improved tolerance. However these agents are expensive and at least one-third of the eligible patients fail to show any useful response. Finding a means to predict those who will respond, and to anticipate relapse are, therefore, of obvious importance.

T helper-type 1 (Th1) lymphocytes orchestrate much of the inflammation in Crohn's disease mainly via production of TNFα, which appears to play a pivotal role as a pro-inflammatory cytokine. It exerts its effects through its own family of receptors (TNFR1 and TNFR2), the end results of which include apoptosis, c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) activation and NF-κB activation. Activated NF-κB enters the nucleus and induces transcription of genes associated with inflammation, host defense and cell survival.

The promoter region of the *TNF* gene lies between nucleotides –1 and –1300, and encompasses numerous polymorphic sites associated with potential binding sites for various transcription factors. Carriers of the *TNF* allele 2 (*TNF2*), which contains a single base-pair polymorphism at the –308 promoter position, produce slightly more TNFα in their intestinal mucosa than non-*TNF2* carriers. *TNF* polymorphisms also appear to influence the nature and frequency of extraintestinal manifestations of inflammatory bowel disease (IBD).

A number of routes of inhibition of TNF are being investigated. Most extensively evaluated is the use of monoclonal antibodies against TNFα (e.g. infliximab). Several large controlled trials indicate that infliximab has a role in treating patients with moderate to severely active Crohn's disease and in fistulating Crohn's disease. Although it would be useful to genetically differentiate 'responders' from 'non-responders,' currently there are few published data on *TNF* polymorphisms in IBD, and often only selected polymorphisms are genotyped. Small studies have shown possible associations between poor response to infliximab and increasing mucosal levels of activated NF-κB, homozygosity for the polymorphism in exon 6 of *TNFR2* (genotype Arg196Arg), positivity for perinuclear antineutrophil cytoplasmic antibodies (ANCA), and with the presence of increased numbers of activated lamina propia mononuclear cells producing interferon-γ and TNFα.

This is a rapidly changing field, and more information of greater direct clinical benefit can be expected soon.

Crohn's disease is a chronic inflammatory condition that affects the gastrointestinal tract anywhere between the mouth and anus – most commonly the distal ileum and colon. Fistulae (abnormal connections between intestine and other epithelial surfaces such as bladder or skin) is present in up to 15% of patients at some stage in their disease.[1] Although the incidence of Crohn's disease is generally lower than that of the other principal inflammatory bowel disease (IBD), ulcerative colitis, it appears to be increasing steadily in most of the regions in which it has been studied sequentially. Collected studies from Europe and North America yield annual incidence figures of between 2 and 6 per 100 000, with rates of around 5 per 100 000 from the more comprehensive population studies performed since the early 1980s.[2-4] Although the cause of Crohn's disease remains obscure, genetic, immunologic and environmental factors contribute to its pathogenesis, and the mucosal immune system is involved.[5,6] Current therapies for patients with Crohn's disease and other forms of IBD are limited by low to moderate efficacy, delayed onset of action, frequent administration regimens, and adverse effects.

Within the disordered immune system of Crohn's disease it appears that monocytes and T helper-type 1 (Th1) lymphocytes orchestrate much of the inflammation of the disease, not least through the production of pro-inflammatory cytokines such as interferon-γ (IFNγ), interleukin (IL)-1β, and tumor necrosis factor-α (TNFα). Following the isolation and characterization of TNFα[7] and its two receptors (TNFR1 and TNFR2),[8] detailed regulatory processes for transcription, secretion, and post-receptor actions of TNF are rapidly being discovered. TNFα[9] plays a pivotal role in Crohn's disease. The recent development of genetically engineered agents that inhibit TNFα has the potential to radically alter the treatment of Crohn's disease by establishing a new therapeutic class with high efficacy, rapid onset of action, prolonged effect, and improved tolerance.[10] Nonetheless, at present these agents are mostly very expensive and are ineffective in around a third of patients. A pharmacogenomic approach to case selection has much to recommend it. Before discussing these aspects in more detail, is worth reviewing what is currently known about TNFα, the genetics of IBD, and the role of TNFα in inflammation in Crohn's disease.

1. The Tumor Necrosis Factors (TNF)

TNF was initially described as a protein capable of killing tumor cells *in vitro*. There are two closely related TNF cytokines: TNFα and TNFβ [the latter is also known confusingly as lymphotoxin-alpha (LTA)]. These cytokines have co-evolved with a family of receptors that are involved (*inter alia*) in programmed cell death, or apoptosis. The TNFs are principal mediators of innate (or natural) immunity. TNFα is a potent pro-inflammatory cytokine which is produced mainly by monocytes and macrophages, but also by T and B lymphocytes.[11] The amount produced depends on the activation state of the cells and the inducing stimuli. Lipopolysaccharide (LPS), found in the cell walls of Gram-negative bacteria, is probably the strongest single inducer of TNFα production, but there are various other stimuli, including other bacteria, viruses, parasites, and neoplasia.

The mature, active form of soluble TNFα is a 51kD homotrimer formed by the association of three 17kD monomers. These monomers originate from a membrane-anchored 26kD precursor (pro-TNF) that is cleaved to the secreted 17kD form by TNFα-converting enzyme (TACE), a member of the metalloproteinase disintegrin family of membrane-anchored glycoproteins.[12,13] The response induced by TNFα is mediated through either one of its receptors, TNFR1 and TNFR2. TNFR1 is expressed by many different cell types and is a member of a family of so-called death receptors, which are closely involved in apoptosis. TNFR2 is more restricted in its expression, mainly appearing on white blood cells. The intracellular portion of TNFR1 contains a 'death domain' which is required for the signaling of apoptosis and NF-κB activation. The aggregation of ligand-independent TNFR1 cytoplasmic domains is inhibited by a silencer of death domains (SODD), which keeps TNFR1 in an inactive, monomeric state. Binding of TNF homotrimers to the extracellular domain of the receptor causes the SODD to dissociate and allows aggregation of the membrane receptors. The aggregated receptors of TNFR1 recruit the adaptor protein TNFR-associated death domain (TRADD). TRADD in turns recruits Fas-associated death domain (FADD), TNFR-associated factor 2 (TRAF2), and receptor interacting protein (RIP) to form the TNFR1 signaling complex. This then activates signaling cascades leading to apoptosis, c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) activation and NF-κB activation. NF-κB then enters the nucleus and induces transcription of genes associated with inflammation, host defense and cell survival.

Activation of TNFR2 by TNF involves recruitment of TRAF2, TRAF1 and the cellular inhibitor of apoptosis proteins (c-IAPs) to the intracellular domain of the receptor. NF-κB and JNK activation is then mediated by the TNFR2 complex by TRAF2[14] (see figure 1).

In addition to the two receptors for TNFα, monocytes and lymphocytes can express a membrane-bound form of TNFα that probably acts as a cell-to-cell activator. In Crohn's disease TNFα is involved in the recruitment of circulating inflammatory cells to sites of mucosal inflammation, induction of edema, activation of coagulation, and promotion of granuloma formation.[13]

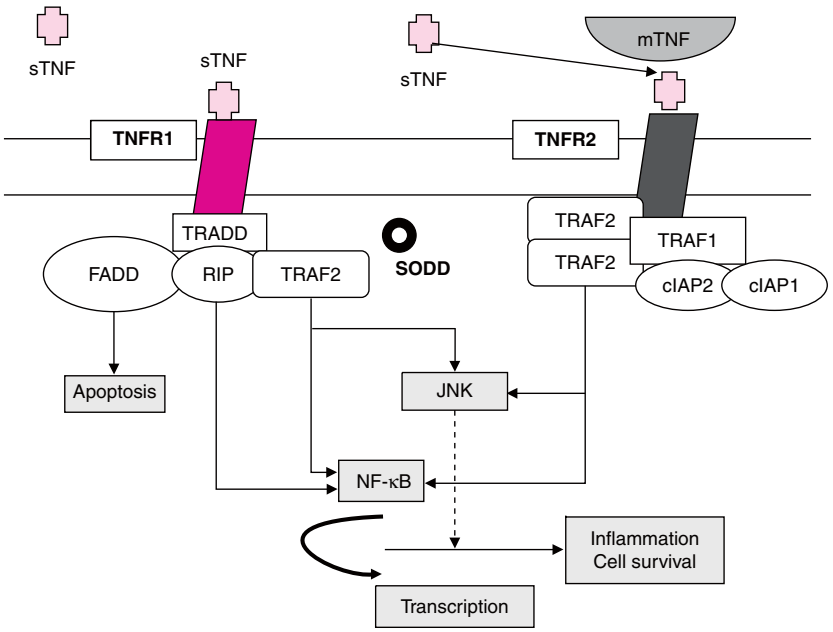

Fig. 1. Signal transduction by tumor necrosis factor (TNF) through TNF receptor (TNFR1) and TNFR2. **cIAP** = cellular inhibition of apoptosis proteins; **FADD** = Fas-associated death domain; **JNK** = c-Jun N-terminal kinase; **RIP** = receptor interacting protein; **SODD** = silencer of death domains; **sTNF** = soluble TNF; **TRADD** = TNFR-associated death domain; **TRAF*x*** = TNFR-associated factor *x*.

It increases the expression of cell adhesion molecules, platelet-activating factor and IL-8 by endothelial cells, and stimulates local production of other pro-inflammatory and chemotactic substances, including IL-1, IL-8 and leukotriene B4. Because of this, TNFα promotes 'rolling' of neutrophils over inflamed endothelium, tight adhesion of neutrophils to the endothelium, and hence neutrophil transmigration. It also has procoagulant effects, and induces the secretion of granulocyte macrophage colony stimulating factor.[15]

Many studies have shown that the concentrations of TNFα, and of other pro-inflammatory cytokines, are increased in the serum, intestinal mucosa and the gut lumen of patients with Crohn's disease.[16,17] Because of its important role in the orchestration of the inflammatory cascade, neutralization of TNFα has been explored as a therapeutic strategy for the management of patients with Crohn's disease.

2. Genetics of TNFα

In humans, the gene for TNF, which contains coding regions for TNFα and TNFβ, is located on chromosome 6, within the major histocompatability complex (MHC). It is approximately 250kb centromeric of the *HLA-B* locus and 80kb telomeric of *HLA-DR*. It consists of 4 exons and 3 introns.

Westendorp et al.[18] studied the heritability of TNF production in monozygotic twins and their first degree relatives using *ex vivo* endotoxin-stimulated whole blood samples. It was estimated that approximately 60% of the variation in TNFα production was genetically determined. Also, HLA-DR3-positive individuals produced more TNF than individuals with HLA-DR2.[19] It is probable that the person-to-person variation in magnitude of the TNF response is largely governed by differences within the *TNF* gene.

2.1 TNF Promoter Polymorphisms

The promoter region of the *TNF* gene encompasses nucleotide positions –1 to –1300. There are numerous polymorphic sites associated with potential binding of numerous transcription factors, including OCT1 at –376, and NF-κB at –803. One of the sites that has been especially investigated is at position –308, which has both cell-type and stimulus-specific regulatory elements, and serves as a potential binding site for numerous transcription factors, including AP-1, AP-2, NF-κB, Ets, SP-1, C/EBPβ, and the cyclic adenosine monophosphate (cAMP) response element (CRE).[14,15,20] These transcription factors are important in the regulation of the TNF gene, and interactions between factors may vary depending on the type of cell or the specific extra-cellular stimulus.

Allele 2 of the *TNF* gene (*TNF2*) contains a single nucleotide polymorphism (SNP) at promoter position –308. Carriers of at least one copy of *TNF2* produce slightly more TNFα in their intestinal mucosa than non-*TNF2* carriers.[21] Furthermore, *TNF2* carriers tend to be over-represented among certain subgroups of patients with Crohn's disease. Patients who are steroid-dependent, those with fistulating rather than stricturing disease, and those with colonic rather than small bowel disease, are statistically more likely to be *TNF2* carriers. This SNP at position –308 might be expected to contribute to the differences seen in response to anti-TNF treatment. Those with a more intense TNFα-driven reaction at the mucosal level (i.e. *TNF2* carriers) ought to be more responsive, but this has not yet been studied.

Transmission disequilibrium studies currently published only in abstract form confirm an association between the –1031T/C polymorphism of the *TNF* promoter gene and Crohn's disease, as opposed to other forms of IBD.[22] No less than 515 parent-child trios were studied, revealing a genotype relative risk for the C allele of 1.5 ($p = 0.01$), and a population attributable risk of around 20%.

The –1031T/C polymorphism and two novel TNF gene polymorphisms, at positions –863C/A and –857C/T, were positively associated with Crohn's disease in a cohort of Japanese patients, and are also claimed to influence not only susceptibility to Crohn's disease but also disease location.[23] Earlier, Higuchi et al.[24] had shown that individuals with this haplotype showed a 1.8-fold higher production of TNFα in response to stimulation of peripheral blood mononuclear cells by Concanavalin A.

A study by Bonen et al.,[25] published only in abstract form, characterized the genomic variation throughout the *TNF* gene in patients with IBD and assessed the functional impact of the suggestive gene variants. The *TNF* gene was sequenced in 12 unrelated patients with IBD who were from families with the greatest evidence for linkage to Ch 6p. SNPs were typed in 135 multiply-affected families and studied using the TDT. Electromobility shift assays (EMSAs) were performed using allele-specific oligonucleotides in this region incubated with nuclear extracts from U937 mononuclear cells stimulated with phorbol myristyl acetate. Three specific DNA-protein complexes were observed, but no allele specific binding was present. Promoter variants at positions –1031, –863, –308 and –238 were identified in patients with IBD. The single locus TDT tests for linkage showed neutral or inverse transmission for the less common –863A and –308A alleles. No significant linkage was associated with the less common A allele at –1031 or –238. So they concluded that no single *TNF* promoter polymorphism by itself is significantly associated with IBD.[25]

There is also evidence that *TNF* polymorphisms influence the nature and frequency of extraintestinal manifestations of inflammatory bowel disease. In a study as yet only published in abstract form,[26] the Oxford group examined 4 common polymorphisms and identified a clear association between polymorphism –1031 of the *TNF* gene and the presence of erythema nodosum; 69 versus 37% in controls ($p = 0.001$). Groups of patients with various forms of arthropathy or uveitis were also studied, although the only robust association identified was between erythema nodosum and the –1031 polymorphism.

It is important to note at this stage however, that there is a lack of published data on TNF polymorphisms in patients with IBD and often only selected polymorphisms are genotyped. Also the findings of association studies and studies relating polymorphisms to TNF function have not been confidently reproduced elsewhere and some cases are conflicting.

2.2 The 3′ Untranslated Region

Post-transcriptional control of TNF mRNA has been localized to the 3′untranslated region of the *TNF* gene, which encodes adenosine-uracil multimers (known as AU-rich elements) that are present in the mature mRNA of TNF as well as other cytokines, proto-oncogenes and growth factors. These elements seem to have an mRNA stabilizing role, and also confer translational repression.[27] On the contrary, although tristetraprolin (TTP), a cytoplasmic protein found in macrophages, also binds to AU-rich elements of the TNF mRNA, it causes the message to destabilize.[28,29] Biosynthesis of TTP is induced by TNF-inducing stimuli, including TNFα itself. TTP-knockout mice display signs and symptoms of chronic TNFα excess (e.g. inflammatory arthritis and cachexia), and these phenomena are prevented by repeated injections of antibodies to TNFα.

The AU-rich elements additionally serve as specific targets for translational activation by JNK, stress activated protein kinase (SAPK), and p38 mitogen-activated protein kinase (MAPK) mediated signals. TNF gene expression can be regulated by cAMP modulation or MAPK (p38, ERK or JNK) inhibition.[30]

3. Anti-TNF Treatment in Patients with Crohn's Disease

The assumed role of TNFα as a pivotal cytokine in the pathogenesis of IBD has made it a target for specific immunotherapy. A number of routes to inhibition of TNFα are being investigated (see table I).

3.1 Inhibiting TNF Production

Pentoxifylline affects the production of TNF by increasing intracellular cAMP concentration.[31] Clinical trials of pentoxifylline have, however, not confirmed any efficacy in Crohn's disease.[32]

Thalidomide inhibits TNF by increasing the degradation of mRNA for TNF.[31] To date there are no properly controlled trials, but the positive data from open studies are reasonably encouraging,[33,34] combined as they are with a whole series of supportive abstracts.

There are no clinically available agents that inhibit TNF production by preventing cleavage of the precursor (pro-TNF), or by blocking the TACE, but preliminary work aiming to inhibit the transcription of TNF exists. Inactivation of NF-κB by an antisense oligo-nucleotide has been examined in animal models, in which its suppression reduced the inflammation of trinitrobenzene sulfonic acid sodium-induced colitis.[35]

3.2 TNFα-Neutralizing Antibodies

Infliximab is a chimeric IgG1 murine-human monoclonal antibody, which neutralizes TNFα by blocking soluble cytokine, and by binding to transmembrane TNFα.[23,29,36] However anti-TNF antibodies do not prevent signaling at the TNF receptor by lymphotoxin (TNFβ). Several large controlled trials indicate that infliximab has a role in the treatment of patients with moderate to severely active Crohn's disease and in fistulating Crohn's disease.[36,37] Similar but more preliminary data exist for the more humanized anti-TNF antibody – CDP571.

Although the short-term safety record of the therapeutic antibodies is improving, concerns about infusion reactions and secondary infections remain, and there is a lack of knowledge regarding the clinical significance of human anti-chimeric antibodies. Moreover, these agents are very expensive (approximately $US2000 per treatment when hospital costs are included as well as the price of the drugs), and at least one-third of the eligible patients fail to show any useful response. Finding means to predict those who will respond, and to anticipate relapse, are of obvious importance.

A study by Nikolaus and colleagues,[38] as yet only published in abstract form, involved 22 patients with acute Crohn's disease treated by a single infliximab infusion. They were refractory to steroids and immunosuppressants and all had severe inflammation. Mucosal levels of activated nuclear NF-κB-p65 decreased within two weeks of infusion in all patients, and 20 of the 22 exhibited a clinical response. As patients relapsed, their levels of TNFα rose, preceded by increases in their levels of activated mucosal NF-κB-p65, 2 to 4 weeks prior to clinical or endoscopic features. It is important to mention that at the 4-week timepoint, which is the point typically used in other clinical studies of infliximab to assess if there has been a response, only 40% of the patients were in response. This shows that response to infliximab is not an all-or-none response, but more of a dynamic process, which is characterized by a different duration of responsiveness to anti-TNF treatment.

Table I. Routes to therapeutic inhibition of TNFα

Blocking TNFα production/secretion
Pentoxifylline
Thalidomide
Blocking TNFα-converting enzyme
NF-κB antisense oligonucleotides
Non specific: corticosteroids, cyclosporine
Monoclonal antibodies directed against TNFα
Infliximab
CDP571
TNF-binding neutralizing fusion proteins
TNFR p75-Fc/etanercept

TNF = tumor necrosis factor; **TNRF** = tumor necrosis factor receptor.

The same group has studied a possible association between TNF receptor genotype and responsiveness to infliximab. Although the data has thus far been published only in abstract form,[39] some useful conclusions could be drawn. Both *TNFR1* and *TNFR2* genes were sequenced in 45 patients, and SNPs were typed by TaqMan®. A novel silent polymorphism in exon 2 of *TNFR2* was identified, together with a coding polymorphism (Met^{196}→Arg; M196R) in exon 6 of the *TNFR2*.[39] Six out of 90 patients who were homozygous for the mutant exon 6 (genotype Arg196Arg), and these patients had a lower response rate (16.7%) than heterozygotes and wild-type homozygotes (63.1%). Despite the small numbers, this reached statistical significance ($p = 0.0036$). The observations were consistent whether Crohn's Disease Activity Index (CDAI)-defined response or full remissions were addressed (0 *vs* 36% for the latter). The silent exon 2 mutation appeared to be in strong linkage disequilibrium with that on exon 6, but a range of other non-coding polymorphisms of *TNFR2* and the *TNF* promoter region was non-informative. There is clearly potential for the use of the exon 2 or 6 mutations in selecting the most suitable patients for infliximab therapy, although prospective studies are warranted.

In another multicenter trial, 73 patients who had responded to a single infusion of infliximab (defined by a reduction of CDAI >70 points) received a further four infusions or placebo at 8-week intervals.[40] Patients who were receiving azathioprine or mercaptopurine before and during the infliximab period had a better

clinical outcome (75% response rate) than those who were not (50%). Whether this is a clinically significant observation is not known as this was a *post hoc* subgroup analysis that was not part of the original study design.

Antineutrophil cytoplasmic antibodies (ANCA) are found differentially in IBD (more in ulcerative colitis than in Crohn's disease, but more in Crohn's disease than in healthy controls).[41-43] The presence of these antigens is at least partially genetically determined, and it appears that they may help to predict response to infliximab. Expression of a particular variant of ANCA (sANCA), which shows a speckled pattern over the entire neutrophil on indirect immunofluorescence staining, has been compared with that of the more characteristic (ulcerative colitis-like) perinuclear staining (pANCA).[23,44] Patients whose neutrophils showed the speckling ANCA pattern formed a subgroup with a better response to infliximab, and those positive for perinuclear ANCA did worse. The same study revealed that homozygotes for the four microsatellites: LTA NcoI-TNFc-aa13L-aa26: haplotype 1-1-1-1, identified subgroups with a poorer response.[45]

Differences in cytokine production between patients who responded to infliximab and those who did not were observed in a subset of patients (defined by ANCA pattern and TNF genotype) treated at Cedars-Sinai Medical Center.[45] Responders had decreased numbers of activated lamina propia mononuclear cells (LPMCs) producing IFNγ and TNFα, in contrast to a non-responder who had increased LPMC IFNγ and TNFα. This finding suggested that clinically effective therapy with infliximab was associated with down-regulation of mucosal Th1 responses in Crohn's disease – again a phenomenon partially under genetic control. However, it must be noted that this was a small study with only 59 patients, and these results must be repeated in a larger series of patients so one can control for type-I statistical errors.

3.3 TNF Receptor Fusion Proteins

Etanercept is a genetically engineered fusion protein that consists of two identical recombinant chains of the human extra cellular TNF-receptor p75 component fused to the Fc domain of human IgG1. It is known to bind TNFα, and, unlike infliximab also to lymphotoxin. Etanercept has been tested with considerable success in patients with refractory rheumatoid arthritis.[46] Despite some preliminary positive data, a well-conducted clinical trial has shown that it is not effective in patients with Crohn's disease.[47] Therefore the efficacy of TNF-binding molecules differs in Crohn's disease and rheumatoid arthritis. This may be in part related to the fact that etanercept works as a cytokine 'carrier' primarily by binding soluble trimers of TNFα with high affinity, and thus competitively inhibiting the binding of such trimers to membrane bound TNFα receptors. The result is that TNFα is rendered biologically inactive. In contrast, infliximab has a strong binding affinity to soluble TNFα trimers as well as to the transmembrane form, resulting in complement fixation and antibody dependent cytotoxicity.[10] Thus the effect of infliximab is mediated by induction of apoptosis in monocytes,[48] which may account for the differences between infliximab and etanercept. The apoptosis is conducted through a caspase-dependent pathway, and Lügering et al. have identified caspases 3, 8, and 9, and regulatory transcriptional activation of Bax and Bak (members of the Bcl-2 family) in the infliximab response. Each of these caspases is subject to genetic polymorphism,[49,50] strengthening the key role of pharmacogenomics in infliximab therapy. Currently no data exist on their individual influences on the response to infliximab or other agents used in the treatment of patients with Crohn's disease.

4. Summary

The action of TNFα in the inflammatory cascade plays an apparently pivotal role in Crohnbs disease. An important SNP at position –308 in the promoter of the TNF gene is associated with increased TNFα production at the mucosal level, and TNF microsatellite polymorphisms are positively associated with Crohnbs disease and its clinical subtypes. Interference with intrinsic TNFα responses via TNF neutralizing antibodies clearly helps many afflicted patients, but there are also poor responders. Understanding the genetic variations in TNF and components of its signaling cascade, as well as individual genetic differences that may influence Crohnbs disease progression, will aid in predicting response to anti-TNF agents and therefore improve the specificity of treatment of this debilitating condition.

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