



Review

Macrophages and neurodegeneration

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Abstract

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS). Demyelination is a classical feature of MS lesions, and neurological deficits are often ascribed to the reduced signal conduction by demyelinated axons. However, recent studies emphasize that axonal loss is an important factor in MS pathogenesis and disease progression. Axonal loss is found in association with cellular infiltrates in MS lesions. In this review, we discuss the possible contribution of the innate immune system in this process. In particular, we describe how infiltrated macrophages may contribute to axonal loss in MS and in experimental autoimmune encephalomyelitis (EAE), the animal model for MS. An overview is given of the possible effects of mediators, which are produced by activated macrophages, such as such as pro-inflammatory cytokines, free radicals, glutamate and metalloproteases, on axonal integrity. We conclude that infiltrated macrophages, which are activated to produce pro-inflammatory mediators, may be interesting targets for therapeutic approaches aimed to prevent or reduce axonal loss during exacerbation of inflammation. Interference with the process of infiltration and migration of monocytes across the blood–brain barrier is one of the possibilities to reduce the damage by activated macrophages. Crown Copyright © 2004 Published by Elsevier B.V. All rights reserved.

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1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS). In the brain and spinal cord of MS patients, inflammatory lesions occur in association with blood vessels. Magnetic resonance imaging (MRI) studies revealed that gadolinium-enhancing lesions appear and disappear, even in clinically stable MS patients, indicating that the formation of new lesions is variable in place and time. Demyelination is a classical feature of MS lesions, and neurological deficits are often ascribed to reduced conduction capacity of demyelinated axons. However, it has already been recognized in the earliest MS studies by Charcot [28] that axonal loss is also an important feature of MS pathology. Nevertheless, in the century of research following these findings, MS was generally regarded as a demyelinating disease in which axons are relatively spared. Only recently, axonal loss has received renewed interest mainly due to the use of techniques detecting ongoing axonal damage. Now it becomes clear that axons are damaged during inflammation in MS which may eventually lead to substantial irreversible axonal loss [49,94,174]. Evidence for the contribution of cytotoxic CD8⁺ T cells to axonal damage in an MHC class I dependent way is evolving [10,88,118,119,145] and mechanisms are being unraveled [111,114,144]. In addition, antibodies to axonal components like neurofilaments and gangliosides (predominantly located in neurons) are elevated in the CSF of MS patients [120,142,151] and may contribute to neuronal damage by directing macrophages to their neuronal and axonal targets. In this review, we will not discuss these antigen-specific contributions of the immune system to axonal loss. Our focus is on the possible contribution of the innate immune system in this process. In particular, we describe how infiltrated macrophages may contribute to axonal loss in MS and in experimental autoimmune encephalomyelitis (EAE), the animal model for MS.

2. Axonal damage in MS and EAE tissue

Axonal loss can be detected by classical histochemical methods such as Bodian silver impregnation or by electron microscopy. The percentage of axon loss is assessed by comparison of total numbers of axons in affected and unaffected tissue. After the early descriptions of Charcot, contradictory results have been reported, some claimed profound (50%) axonal loss [28] whereas others described unaltered axonal densities in most MS lesions [69]. These discrepancies were explained by differences in sensitivities of used methods, leaving the exact percentage of axonal loss unresolved. Recent, more detailed, studies show that there is indeed progressive axonal loss in MS lesions: up to 70% of axons can be lost in the spinal cord of MS patients [12]. Axonal loss correlates with neurological disability in MS

[37,43,100] and chronic EAE [183], suggesting that axonal degeneration is at least partly responsible for disease progression. The classical methods, such as silverstaining on postmortem tissue, detect axonal loss that may be caused months or even years before. To fully understand the underlying mechanisms of axonal loss, it is important to detect axonal damage at an early stage.

Nowadays, the accumulation of the amyloid precursor protein (APP), a normal neuronal membrane-spanning glycoprotein transported by fast axonal transport [89], is used as a marker for acute axonal damage in the CNS [18,59,97,137,155,185]. In MS, the “age” of a lesion is indicated by various criteria, distinguishing early and late active lesions, both with signs of ongoing demyelination and inflammation, and inactive lesions [40,175]. Accumulation of APP was detected in axonal ovoids in active lesions and at the border of inactive lesions [49]. APP accumulation was more frequent in early and late active lesions compared to relatively ‘older’ inactive lesions in MS, but was unrelated to lesion age in EAE. In both EAE and MS studies axonal damage correlated with the location of cellular infiltrates [43,49,90,94].

Immunohistochemistry for non-phosphorylated neurofilaments is another method to detect axonal damage in MS lesions which was first described by Trapp et al. [174]. In healthy axons, phosphorylated neurofilaments are predominantly found in axons, whereas non-phosphorylated neurofilaments are mainly localized in perikarya and dendrites [162]. Using this method, transected axons, caliber alterations and terminal ovoids have been identified in MS lesions. Also, discontinuous non-phosphorylated neurofilament immunoreactivity was observed, which may indicate axonal degeneration distal from the sites of transection, as in Wallerian degeneration [22,174]. Whereas APP is confined to short axonal segments, non-phosphorylated neurofilament staining is more diffuse and uniformly distributed along the damaged axon [104].

Recently, changes in dendritic and synaptic proteins have been found in EAE, indicating loss of contacts between neurons [189]. Besides this, a redistribution of ion channels in axons indicated neuronal deficits [31,32,141]. Due to demyelination, an increased expression or (re)distribution of certain types of ion channels is induced which may temporally restore axonal conduction. Eventually, this ion channel redistribution may have a deleterious effect causing an influx of noxious levels of Ca²⁺ and subsequent axonal destruction [13,16,17,55]. In EAE, increased expression of specific types of sodium channels co-localized with APP immunoreactivity and the presence of a Na⁺/Ca²⁺ exchanger [32,33]. The immunostaining for the pore-forming unit of the voltage-gated Ca²⁺ channel (N-type) was also increased in demyelinating lesions and co-localized with APP positivity, indicating altered Ca²⁺ transport [91]. In addition, expression of genes involved in Ca²⁺ homeostasis was found to be dysregulated during EAE, indicating early neuronal dysfunction [122].

In MS patients, magnetic resonance spectroscopy (MRS) has now been established as an important tool to detect ongoing demyelination and axonal damage. With proton MRS, changes in *N*-acetylaspartate (NAA) levels can be measured [37,107]. NAA is an excitatory amino acid, primary present in neurons and neuronal processes [11]. The exact function of NAA is unknown, although it has been suggested to play a role in the regulation of protein synthesis, neurotransmitter metabolism and in protecting neurons from osmotic stress [9,170]. A decrease in NAA levels, in relation to creatine levels, is used as a marker for axonal and neuronal damage in tissues [157]. NAA levels are decreased in MS lesions as well as in the surrounding normal-appearing white (NAWM) [48,56,108] and gray matter [50]. Recent publications report that a decrease in NAA occurs very early in the course of MS and may be reversible [36,42,44,108]. On MRI images, the so-called hypointense T1 lesions are associated with tissue destruction and axonal loss [21,178]. Both decreased NAA levels as well as T1 enhanced lesions correlate with increased disability [37,38,41,42,70,129].

3. Macrophages in the central nervous system

In MS, neuronal dysfunction has been associated with the presence of inflammatory cells in the CNS [49,174]. However, the exact role of these inflammatory cells in the process of axonal loss is yet unknown. Cellular infiltrates may contribute to axonal loss, reflect a response to axonal damage, or even exert protective effects on neurons. For elucidation of the role of inflammation in axonal damage and repair, animal studies are indispensable. EAE provides a model for cellular infiltration in the brain with concomitant neurological deficits [83,131,183]. We have previously shown that depletion of macrophages in acute EAE leads to a complete suppression of clinical signs [83]. Since the extent of demyelination is restricted in the acute EAE model [131], this beneficial effect cannot be explained by the contribution of macrophages to demyelination, but rather indicates a direct effect of macrophages on axonal function.

In peripheral tissues, macrophages exhibit considerable heterogeneity with respect to their receptor expression and pathways leading to activation. Gordon [68] distinguishes at least 5 types of activated macrophages, among which the classical route of activation by lipopolysaccharide or interferon (IFN)- γ , leading to production of inflammatory mediators such as nitric oxide (NO) and interleukin (IL)-12. The classically activated macrophages have pro-inflammatory and cytotoxic capacities. On the other hand, the so-called alternatively activated macrophages can be induced by IL-4 and glucocorticoids. Alternatively activated macrophages have an increased expression of arginase-1 and various neurotrophic factors, and are thereby more protective [105,117].

In the CNS, equivalents of these distinct populations have not yet been established but infiltrated macrophages in MS and EAE lesions display at least for a certain period of time a classically activated phenotype, since they express inducible nitric oxide synthase (iNOS) [39] and MRP8/14, a marker for activated macrophages [19,20]. These infiltrated macrophages may contribute to axonal damage by their production of pro-inflammatory mediators.

In the remaining part of this review, we will focus on the potential detrimental role of infiltrated macrophages in neurodegeneration and loss of axonal function.

4. Inflammatory mediators: innate mechanisms contributing to axonal loss

Macrophages have various means to eliminate unwanted cells or pathogens from the body. An important tool for this is the production of mediators that cause death of cells or microorganisms. In MS and EAE, macrophages infiltrate the CNS, where they produce locally after mediators. CSF derived from MS patients in a chronic disease stage induces axonal damage in vitro [2], indicating that soluble mediators, secreted during the course of MS, are able to induce damage to neurons directly. In addition, macrophage conditioned medium is reported to be detrimental to neurons in vitro [52,134,135]. Conversely, others report no [4], or even a beneficial effect [79,112] of macrophages on cultured neurons. Here we describe possible effects of products of activated macrophages such as pro-inflammatory cytokines, free radicals, glutamate and metalloproteases, on axonal integrity.

4.1. Pro-inflammatory cytokines

Macrophages produce pro-inflammatory cytokines like TNF- α , IL-1 and IL-6. TNF- α was described to be neurotoxic in vitro, especially in neuron–glia cocultures in combination with other cytokines like IFN- γ and IL-1 [26,27,65,76,85,169]. TNF- α increases axonal vulnerability by enhancing demyelination via oligodendrocyte killing [153]. While some of the detrimental effects of TNF- α were explained by induction of NO production, others related it to the potentiation of glutamate excitotoxicity [25]. TNF- α -deficient mice had significant higher numbers of preserved axons after the induction of Wallerian degeneration [156], a neurodegenerative process in which TNF- α and IL-1 are involved [154]. In MS, no correlation could be detected of axonal damage or disease severity with TNF- α expression in the CNS [10] or in serum [92]. Remarkably, in EAE anti-TNF- α antibodies were found to be beneficial while they were detrimental in MS [6,177]. These results make it difficult to elucidate the exact role of TNF- α and the other cytokines in neuroinflammation and induction of neuronal damage [61].

A role for cytokines in initiation of glutamate-induced neuronal damage is also reported for IL-1 β and IL-6 in vitro [140,147]. However, contradictory results are obtained concerning the effects of cytokines on neuronal function in vivo. Several studies indicate that IL-6 may have a neuroprotective effect [15,57,71,113,173]. In these studies, neuronal injury seems to lead to a release of IL-6 and not vice versa.

4.2. Free radicals

Another group of potentially neurotoxic mediators are reactive oxygen species (ROS) and NO. ROS, including superoxide, hydroxyl radicals [75] and hydrogen peroxide, are highly reactive molecules, involved in various signal transduction cascades [78,123]. ROS form little threat in low concentrations as cells possess various defense and repair mechanisms for minor oxidative stress [64]. However, during inflammation, they are released in high concentrations by the oxidative burst of macrophages. High ROS concentrations overrule cellular defense mechanisms and cause oxidative damage to proteins, lipids and nucleic acids. Markers of oxidative stress were elevated in sera of MS patients [8,67,115] and in the CNS of EAE animals [84,103]. In MS patients, ROS were shown to induce DNA damage leading to neurodegeneration [101,179]. Evidence for the crucial role of ROS in EAE is derived from experiments in which specific ROS scavengers were administered. Antioxidants like lipoic acid [106] and bilirubin [99] ameliorate acute EAE by reducing inflammation and axonal damage. Also, endogenous antioxidants or oxidative stress response proteins like catalase [148], metallothioneins [132,133], uric acid [82,87,160], SOD [74] and hemoxygenase-1 [23,46] are protective during neuroinflammation. We have recently studied the effects of flavonoids, which are potent dietary antioxidants, on acute and chronic EAE. Especially the flavonoid luteolin was effective and suppressed the clinical signs of both acute and chronic EAE even when administered after disease onset [77]. Another reactive inflammatory product is NO, synthesized by the enzyme NOS that exists in three isoforms. The neuronal (nNOS) and endothelial (eNOS) isoforms are constitutively expressed and calcium-dependent. The inducible (iNOS) isoform is expressed following de novo enzyme synthesis and is calcium-independent. The latter is induced during inflammation by, e.g., pro-inflammatory cytokines [113]. NO is very reactive and forms products like peroxynitrite and 3-nitrotyrosine. Peroxynitrite (ONOO⁻) is formed by the reaction of NO with superoxide whereas 3-nitrotyrosine is the product of nitration of tyrosine residues by peroxynitrite. NO products are detectable in serum and CSF of MS patients [66], are formed early in EAE, correlate with disease severity [176] and damage myelin and axons [172]. NO induces neuronal apoptosis, especially when neurons are deprived of neurotrophic factors

[47]. iNOS and NO products are mainly present in macrophages and microglia in active MS lesions [5,14,39,127,176] and EAE [81,103]. Evidence for the contribution of NO to axonal damage comes from studies in which axonal conduction was found to be temporally blocked by NO in vitro [143]. In these experiments, NO treatment induced neurodegeneration when axons were activated by electric impulses at physiological frequencies [159]. Demyelinated axons were even more vulnerable to this toxic insult of NO [143]. Sodium and calcium entry blockers protected axons from this NO-induced damage in vitro [86] in vivo during ischemia [166,167] and in EAE [102]. The detrimental effect of NO on neurons may be exerted via inhibition of mitochondrial respiration leading to energy failure, increase in intracellular sodium and calcium concentrations and finally cell death [58,86,167]. This is supported by a study in which both NO and peroxynitrite impaired mitochondrial function by inhibiting components of the respiratory chain [163].

The idea that NO is detrimental during CNS inflammation is further supported by treatment of EAE with aminoguanine, a NOS inhibitor, which decreased axonal necrosis [146]. In addition, it has been reported that MS lesions with iNOS positive cells displayed low axon density, although no correlation was found with APP immunoreactivity [10]. In contrast, others report a protective role for NO in EAE [149,184], possibly caused by inhibition of cellular infiltration into the CNS [93]. These data suggest that NO is involved in various mechanisms in MS and EAE, some being protective and others being detrimental [30,124,130,182].

4.3. Glutamate

Glutamate is the most abundant excitatory neurotransmitter in the CNS. An excess of glutamate causes excitotoxicity in neurons resulting in cell death [29,110,126]. Oligodendrocytes have been found to be especially vulnerable to high glutamate levels in vitro [96] as well as in vivo [109]. Prolonged activation of neurons by glutamate may be damaging through the production of NO, ROS, arachidonic acid, phospholipase A₂ and proteases like calpain causing a calcium influx [80]. Glutamate may also inhibit the expression of an ion pump involved in Ca²⁺ extrusion [122]. Besides this, the important role of glutamate excitotoxicity in CNS inflammation was shown in EAE where treatment with glutamate receptor antagonists resulted in reduced clinical symptoms and axonal damage [138,158]. Recently, an inhibitor of glutamate transmission was described to have similar protective effects in EAE [63]. In the CSF of MS patients, glutamate levels were elevated and correlated with disease severity [7,152,165]. In addition, the expression of glutamate receptors in the CNS of MS patients was reported to be altered and the presence of a specific glutamate receptor correlated with the presence of axonal damage in MS [60]. An excess of glutamate may be

caused via several mechanisms. The neurotransmitter is secreted in large quantities by macrophages [135]. In MS, macrophages and microglia were immunoreactive for the glutamate-producing enzyme glutaminase and co-localized with dystrophic axons [181]. An excess of extracellular glutamate may also be caused by impaired glutamate clearance and degradation by astrocytes and oligodendrocytes [73,125,139,181]. Glutamate clearance is inhibited by inflammatory mediators like the pro-inflammatory cytokines $TNF\alpha$ and $IL1\beta$ [26,51,139,168], ROS [136,180] and arachidonic acid [187].

In conclusion, glutamate can be produced by macrophages, and when present in excessive amounts, can

lead to neuronal damage and degeneration during CNS inflammation.

4.4. *Proteases*

Other players involved in disease processes in MS and EAE may directly or indirectly have an effect on neuronal function. MMPs are produced and secreted by inflammatory cells to degrade the extracellular matrix to facilitate their migration into the CNS. In MS, MMPs are found in acute lesions [3,72,95,98] where they may directly cause axonal transection [24,62,121]. A direct role for MMP-9 in neuronal dysfunction has been reported [186]. The

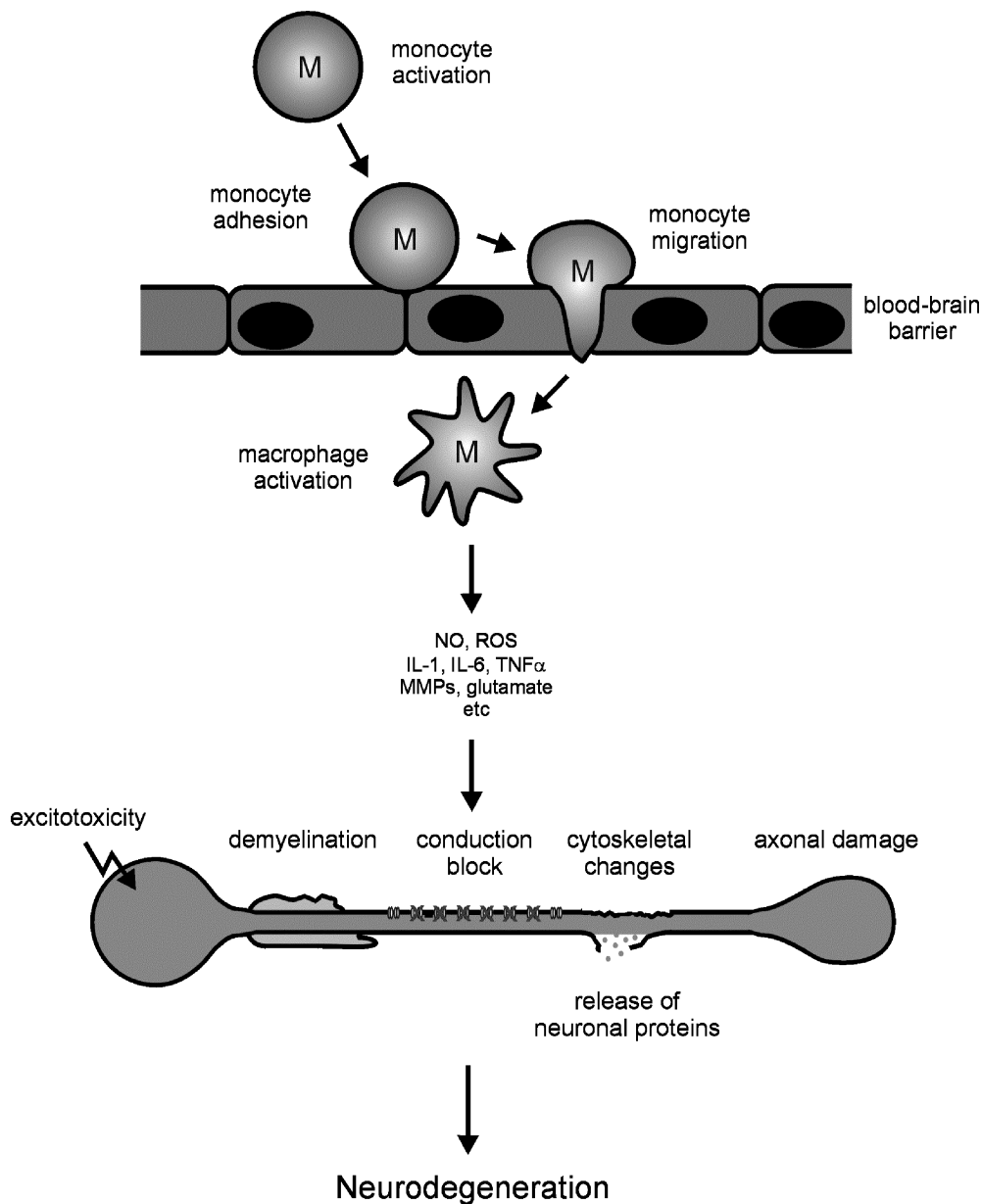


Fig. 1. Schematic drawing of the various stages of monocyte/macrophage contribution to the inflammatory process and to neuronal damage in MS and EAE. Further explanation is found in the text, of which this figure is a summary.

protease tissue plasminogen activator (tPA), increased in MS and EAE [34,102,128,171], is secreted by macrophages and induces neuronal apoptosis in vitro [52,53]. tPA $-/-$ mice show delayed demyelination and axon degeneration after EAE induction [102]. Thus, proteases may play an important role in the induction of axonal damage.

5. Reduction of macrophage infiltration: an attractive target to reduce axonal damage in MS?

The above mentioned data summarized in Fig. 1 suggest that macrophages are an interesting target for therapies aimed at prevention of reduction of axonal damage in MS. Preventing monocyte migration across the blood-brain barrier in one of the possible levels of intervention.

Molecular mechanisms that specifically mediate monocyte transmigration through cerebrovascular endothelium are not as well known as those for lymphocyte adhesion and migration. For monocyte migration across brain endothelial barriers, an important role for VLA-4/VCAM, and PECAM/PECAM interaction, both molecules of the immunoglobulin superfamily (IgSF), has been demonstrated [54]. Also, other IgSF members, like CD47, have a part in monocyte infiltration into the CNS. CD47 is also known as integrin-associated protein, which mediates the final post-adhesion step of monocyte migration into the CNS [45].

One of the emerging therapies for MS is aimed at the interference of the migration of inflammatory cells into the CNS at the level of the BBB. Recently, a humanized monoclonal antibody against the $\alpha 4$ chain of the VLA-4 integrin was developed, called Natalizumab. Treatment with Natalizumab had a significant effect on the primary outcome measure of MS lesion formation, i.e., reduction in the number of newly formed brain lesions established by gadolinium enhancement MRI over the 6-month treatment period, and significantly reduced the number of clinical relapses [35].

Current and future treatments of neuroinflammatory diseases, like multiple sclerosis, aim at dampening the inflammatory cascade in the CNS, and in fact have inhibitory effects on macrophage infiltration. For instance, interferon beta treatment leads to the reduction of new lesions as assessed by MRI [164]. The inhibition of new lesion formation appeared to be due to reduced cellular infiltration, possibly as a result of reduced expression of adhesion molecules on the brain endothelium, as assessed in EAE animals and in brain endothelial cells (ECs) in vitro [54]. Antioxidants like flavonoids profoundly inhibited monocyte migration across the blood-brain barrier (BBB) in vitro and in vivo [77]. Cannabis, now under research as a potential beneficial therapeutic for MS patients, reduced spasticity and clinical signs in EAE [1,188]. Cannabinoids may influence the migration of monocytes across the BBB [150]. Lovastatin, a potent inhibitor of 3-hydroxy-3-methyl

glutaryl coenzyme A (HMG-CoA) reductase, a key enzyme in the cholesterol biosynthesis pathway, may be a promising new therapeutic tool. Lovastatin was shown to suppress the clinical course of EAE by inhibiting monocyte infiltration into CNS [161]. Even sunlight influences monocyte activation, through a bioactive metabolite of vitamin D, 1,25-dihydroxyvitamin D₃. Treatment of EAE animals with this bioactive hormone also decreased macrophage accumulation into the CNS [116].

In conclusion, macrophages probably play an important role in the induction of axonal damage. The identification of the players involved, like molecules that are specifically involved in the entry of monocytes across the blood-brain barrier, is of importance to design brain-specific anti-inflammatory strategies. Also, interference with macrophage-derived mediators, which are involved in axonal damage, is an interesting approach, in particular to reduce axonal damage during acute exacerbations of the inflammatory process in the CNS. The development of therapeutic strategies to prevent axonal damage in MS and EAE will also be valuable for other neuroinflammatory conditions such as ischemia.

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