

Alpha 2-macroglobulin synthesis in interleukin-6-stimulated human neuronal (SH-SY5Y neuroblastoma) cells

Potential significance for the processing of Alzheimer β -amyloid precursor protein

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Cultured human neuronal (SH-SY5Y neuroblastoma) cells synthesize and secrete the potent protease inhibitor alpha 2-macroglobulin (a2M) upon stimulation with interleukin-6 (IL-6) indicating that alpha 2-macroglobulin behaves as an acute-phase protein in the human central nervous system. Exogenous addition of a2M to the cultured neuronal cells resulted in only a slight inhibition of Alzheimer β A4-amyloid precursor protein (APP) synthesis, but markedly inhibited its secretion pointing to the possibility that a2M may affect the proteolytic APP processing. Evidence is provided that IL-6 and a2M are involved in Alzheimer's disease pathogenesis.

Neuron; Alpha 2-macroglobulin; Interleukin-6; Amyloid precursor protein; Alzheimer's disease

1. INTRODUCTION

Many biological processes are initiated by the activities of proteolytic enzymes [1]. Proteases are under the control of a number of protease inhibitors [2]. In the central nervous system, important events such as neural cell migration during brain development and neurite outgrowth after brain tissue damage have been found to depend on a precarious balance between proteases and their inhibitors [3]. Proteases and their inhibitors may contribute to pathogenic events. In Alzheimer's disease (AD), proteases and their inhibitors seem to be of particular importance: (i) the proteases cathepsin B and D [4] as well as the proteinase inhibitor alpha 1-antichymotrypsin [5] have been found to be constituents of AD senile plaques; (ii) two forms (APP₇₅₁ and the APP₇₇₀) of the Alzheimer β A4-amyloid precursor protein (APP) are Kunitz-type proteinase inhibitors [6,7, for reviews see 7,8]; (iii) by investigating APP synthesis in cultured cells it has been found that secretion of (the large N-terminal part of) APP is the result of a proteolytic cleavage C-terminal to residue 16 of the β A4 part of the mature transmembrane APP [9–13].

In AD, APP is degraded by an unknown mechanism to the 42–43 amino acid β A4 molecule which has a high tendency to aggregate and thus contributes to the for-

mation of AD senile plaques [32]. If the above-mentioned secretory cleavage of APP inside its β A4 part [12,13] is part of the normal APP processing, formation of the β A4 protein would be prevented under normal conditions. It appears conceivable that the formation of the pathogenic β A4 protein in AD may be the result of an inhibition of the APP-cleaving protease (which is called secretase).

We decided to screen cultured human neuronal cells for the synthesis of a number of proteinase inhibitors. In this communication, we report synthesis of alpha 2-macroglobulin (a2M), the most potent of the known human proteinase inhibitors [14], by interleukin-6 (IL-6)-stimulated neuroblastoma cells. Evidence is provided that IL-6-induced a2M synthesis may be involved in the pathogenesis of Alzheimer's disease.

2. MATERIALS AND METHODS

2.1. Materials

RPMI 1640 medium was from Seromed (Berlin, Germany), supplements (L-glutamine, antibiotics and vitamins) and fetal calf serum (FCS) were from Gibco (Karlsruhe, Germany). Purified murine Nerve growth factor β was a generous gift from U. Otten, University of Basel, Switzerland. Human recombinant interleukin-6 (Boehringer, Mannheim, Germany) had a specific activity of 2×10^6 units/mg as determined by the 7TD1-hybridoma growth assay, equivalent to 1×10^5 units/mg if determined as B-cell stimulatory factor (BSF-2) units. Indication of IL-6 units in this paper refer to BSF-2 units. Human alpha 2-macroglobulin was from Boehringer (Mannheim, Germany). Retinoic acid and dibutyl-cAMP were from Sigma (Deisenhofen, Germany). Protein-A-Sepharose was from Pharmacia

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(Freiburg, Germany). [35 S]methionine (800 Ci/mmol) was from Amersham (Braunschweig, Germany). Protosol was from New England Nuclear (Boston, MA). X-ray films (XAR5) were from Kodak.

2.2. Antibodies

Anti-alpha-tau and anti-neurofilament 200 antibodies were from Boehringer (Mannheim, Germany). Monospecific polyclonal antibodies raised in rabbit and directed against human alpha 2-macroglobulin (a2M) has been described [15]. A monospecific polyclonal antibody directed against the complete recombinant β A4 amyloid precursor protein₆₉₅ (APP₆₉₅) fused to the Fd region of the murine IgM B1-8 gene was raised in rabbit and has been described before [11]. This antibody recognizes all 3 APP forms (695, 751, 770).

2.3. Cell cultures

The SH-SY5Y neuroblastoma clone [16] was a gift from Dr J. Biedler, Memorial Sloan-Kettering Cancer Center (New York, N.Y.). Cells (0.5×10^6 /dish) were grown on 40 mm plastic dishes (Nunc, IL.) in RPMI 1640 medium/1% FCS supplemented with L-glutamine, antibiotics, and vitamins. Murine nerve growth factor β (NGF β), retinoic acid, and dibutyryl cAMP (db cAMP) were added to the medium at a final concentration of 20 ng/ml, 10 μ M, and 0.5 mM, respectively. These components have been described to result in a differentiated phenotype of cultured neuronal cells [17].

2.4. Immunocytochemical staining

SHSY5Y-cells were plated on thermanox plastic coverslips (Nunc, IL.) and cultivated for 5 days as described above. The coverslips were then washed with phosphate-buffered saline (PBS) and fixed with 4% formalin/PBS. After incubation with a 1:10 dilution of the primary antibodies anti-alpha-tau or anti alpha-neurofilament 200, cells were treated with the Vectastain ABC-peroxidase detection system (Vector, Burlingame, CA). Cells were mounted in glyceringelatine (Merck, Darmstadt, Germany) and viewed with a Zeiss III RS microscope.

2.5. Radiolabeling and immunoprecipitation

Radiolabeling and immunoprecipitation was done as described earlier in detail by ourselves [18]. Briefly, cells were metabolically labeled by replacing media by methionine- and serum-free RPMI 1640 medium and addition of [35 S]methionine (100 μ Ci/dish). Cells were allowed to incorporate the radioactive amino acid into newly synthesized proteins for 4 h. For pulse-chase experiments, cells were labeled with 200 μ Ci/dish for 20 min followed by addition of an excess of unlabeled methionine (2 mM final concentration). For immunoprecipitation, media were separated from the cells. Cells were homogenized in 1 ml 25 mM Tris-HCl, pH 7.5, 20 mM NaCl, 1% Natriumdeoxycholate, 1% Triton X-100. Non-soluble material was removed by centrifugation. Cell homogenates and media were diluted with 2 volumes of immunoprecipitation buffer (20 mM Tris-HCl, pH 7.6, 0.14 M NaCl, 5 mM EDTA, 1% Triton X-100). Total de-novo synthesized proteins were determined by TCA-precipitation of 20 μ l aliquots of cell homogenates and media according to [19]. Specific proteins were extracted from equivalent amounts of total TCA-precipitable radioactivity (usually 0.5×10^6 cpm from media). In the case of pulse-chase experiments, specific proteins were extracted from total medium and from one third of the cell homogenate. Prior to extraction with specific antibodies, unspecifically-binding material was absorbed by a sham extraction with rabbit non-immune control serum. After addition of an excess of antibody (usually 10 μ l), the immune complexes were bound to an excess (7 mg) of Protein A Sepharose CL-4B, sedimented, and washed (twice in immunoprecipitation buffer, twice in 50 mM sodium phosphate buffer, pH 7.5). The immune complexes were dissociated by treatment at 95°C for 5 minutes in 35 μ l of 0.1 M Tris-HCl, pH 6.8, 5% 2-mercaptoethanol, 5% sodium dodecylsulfate, 10% glycerol, subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to [20], and fluorography according to [21]. The specific radioactive bands were cut out from the gels, solubilized

with protosol/water (9:1), counted in a liquid scintillation counter, and could be related to total protein synthesis.

3. RESULTS

Human SH-SY5Y neuroblastoma cells were cultured as described in section 2. The cells not only showed marked neurite outgrowth but also stained for the neuron-specific proteins alpha-tau (Fig. 1A) and neurofilament 200 protein (Fig. 1B).

In these cells, we investigated de-novo synthesis of alpha 2-macroglobulin (a2M) by immunoprecipitation from culture media after metabolic labeling. All experiments were reproduced at least 3 times; typical experiments are shown. Spontaneous a2M expression in the human neuronal cultures was very low: if expressed as percentage of total secreted proteins, a2M amounted to about 0.01%. However, de-novo synthesis and secretion was dramatically induced upon incubation of the neuronal cells with interleukin-6 (IL-6) in a time- and dose-dependent manner (Fig. 2). Maximally induced a2M secretion was observed after a 24 h incubation time with IL-6 in a final concentration of 200 units/ml. Then, a2M secretion had increased 20 times and now amounted to 0.2% of total secreted proteins. Secretion on this level in IL-6-stimulated cells persisted for at least 72 h (not shown). Other inflammatory cytokines such as interleukin-1 β (IL-1), interleukin-2, tumor necrosis factor alpha (TNF), gamma interferon, leukemia inhibitory factor (LIF) or transforming growth factor β did not affect a2M synthesis. On the other hand, IL-6 in our neuronal cells did not induce the synthesis of other proteinase inhibitors such as alpha 1-antichymotrypsin or alpha 1-antitrypsin.

Confirming a previous report [22], our SH-SY5Y neuroblastoma cultures constitutively expressed Alzheimer's β A4-amyloid precursor protein (APP) as shown by a pulse-chase experiment (Fig. 3). APP is synthesized intracellularly in several molecular weight forms in the range between 98 kDa and 140 kDa (Fig. 3, lanes 1,5,9). This heterogeneity has previously been shown to be due to the facts first that the APP hn mRNA is differentially spliced into 3 major mRNA forms [6,9,23], and second that the APP protein is subjected to intracellular processing including N-, O-glycosylation, and thiorosine sulfation [11]. Secretion of the major N-terminal part of APP is achieved by proteolytic cleavage of the mature transmembrane APP molecule [12] resulting in secreted APP forms in the molecular weight range between 98 kDa and 123 kDa (Fig. 3, lanes 4,8,12). Treatment of the neuronal cells with IL-6 for up to 3 days did neither affect APP synthesis nor its secretion (Fig. 3, lanes 4-8 compared to lanes 1-4). Treatment of the cultures with exogenously added a2M slightly affected intracellular a2M synthesis (78% of control after standardization with respect to total protein synthesis). However, APP secretion was strongly inhibited (17% of control).

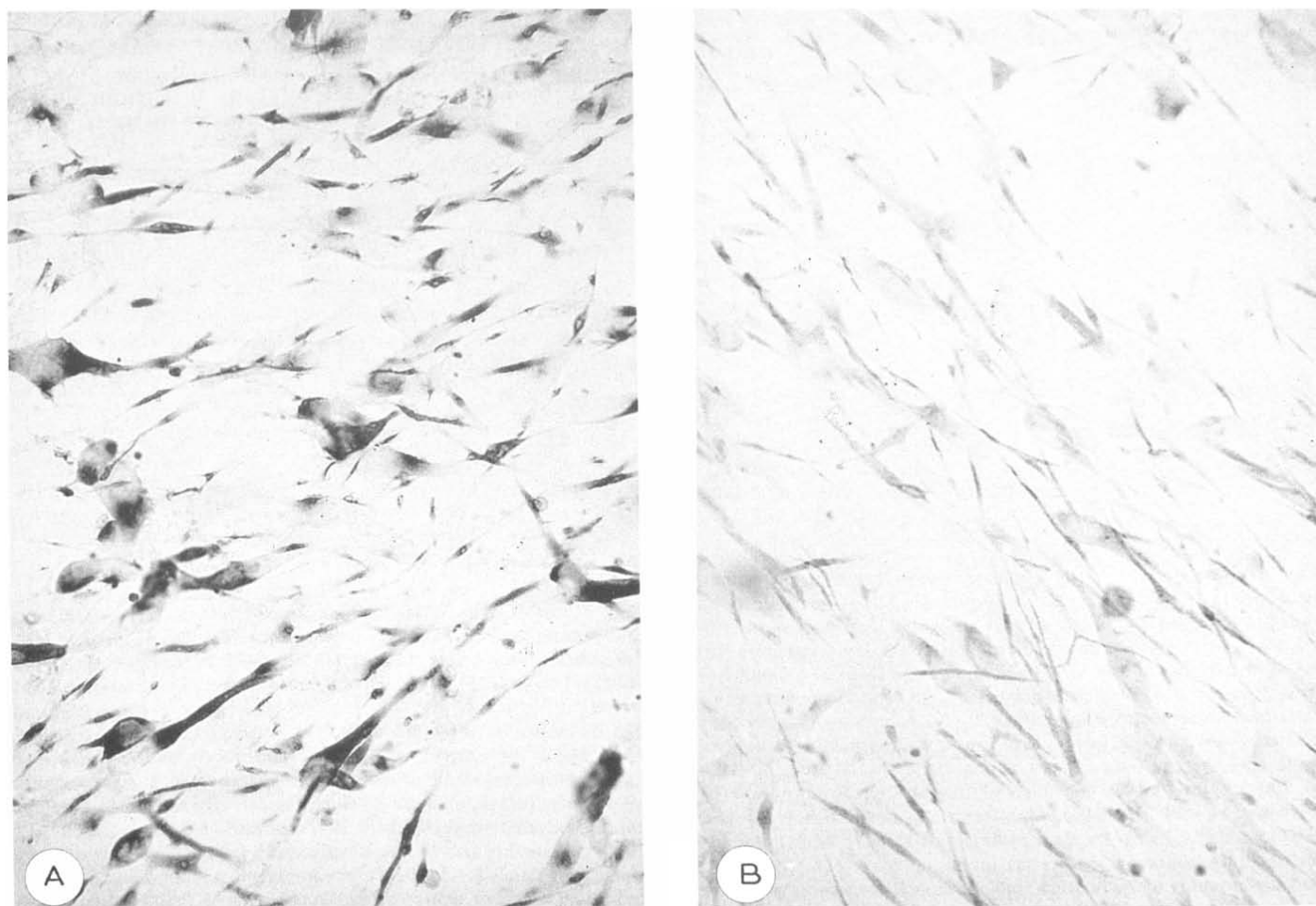


Fig. 1. Morphological characterization of cultured human neuronal (SH-SY5Y neuroblastoma) cells. Human SH-SY5Y neuroblastoma cells were plated on plastic coverslips and cultivated for 5 days as described in section 2. Cells were washed, fixed, subjected to immunohistochemical peroxidase staining with anti-alpha-tau (A) or anti-neurofilament 200 antibody (B), and viewed (magnification $\times 375$). Control stains with non-immune sera showed no staining at all (not shown).

4. DISCUSSION

We found strong induction of alpha 2-macroglobulin (a2M) synthesis in cultured human neuronal cells upon stimulation with interleukin-6 (IL-6). If a2M was exogenously added to cultured neuronal cells, synthesis of Alzheimer's β A4 amyloid precursor protein (APP) was slightly repressed, however, APP secretion which requires a proteolytic cleavage of APP by a still unknown protease (secretase) was strongly repressed. Since IL-6 could induce a2M secretion in the cultured cells, and, on the other hand, a2M could affect APP synthesis and secretion, one would expect that treating the cultures with IL-6 should have an indirect effect on APP synthesis and secretion. However, a2M secreted into the culture medium by IL-6-stimulated cells reaches concentration magnitudes below that of exogenously added a2M which was 1 mg/ml, similar to a2M serum concentrations (1.3 mg/ml). We recently observed marked a2M immunoreactivity in large neurons and in senile

plaques of AD brains (B. Volk, J. Bauer, in preparation). If in fact a2M synthesis and secretion is turned on in vivo, local pericellular a2M concentrations in the brain may well reach significant concentrations.

Our findings could be of considerable biological significance. IL-6 is one of several inflammatory cytokines which are synthesized by the host upon injury or infection [24-26]. Along with TNF and IL-1, this mediator initiates a complex reaction of the host called acute-phase response. In addition to demonstrating for the first time a2M synthesis by neuronal cells we provide evidence that, at least in the central nervous system, human a2M may behave as a cytokine-inducible acute-phase protein. Outside the CNS, human a2M is constitutively present in the serum (serum concentration 1.2 mg/ml [27]) and does not behave as an acute-phase reactant [28]. Until now, systemic acute-phase properties of a2M have only been described in the rat [29,30].

A2M is the most potent protease inhibitor in man

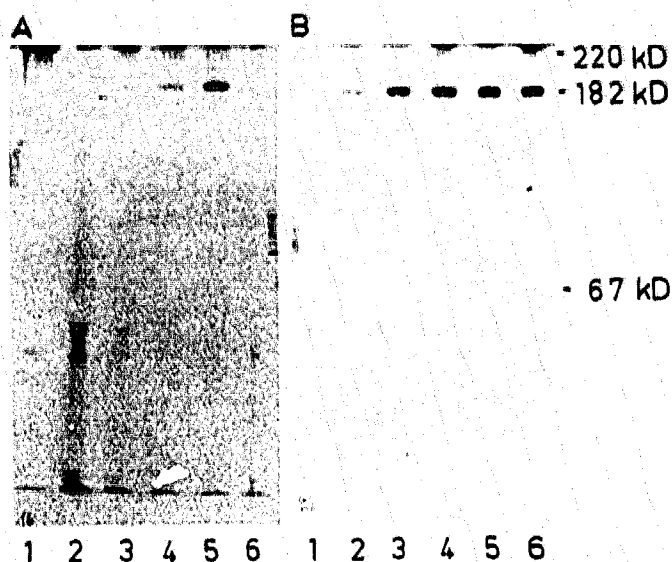


Fig. 2. Induction of alpha 2-macroglobulin synthesis in human neuronal cells by interleukin-6. Human SH-SY5Y neuroblastoma cells (0.5×10^6 cells/40 mm dish) were cultivated for 5 days as described in section 2. (A) Human recombinant interleukin-6 (10 units/ml) was added to the cultures for 6 (lane 3), 12 (lane 4), or 24 h (lanes 5,6) prior to metabolic labelling of the cells. Cells kept in the absence of interleukin-6 served as control (lane 2). (B) Alternatively, cells were treated for 24 h with different concentrations of interleukin-6: control (lane 1), 1 unit/ml (lane 2), 10 units/ml (lane 3), 100 units/ml (lane 4), 200 units/ml (lane 5), 400 units/ml (lane 6). Cells from (A) and (B) were metabolically labeled for 4 h by replacing media with serum- and methionine free RPMI-medium containing $100 \mu\text{Ci}$ [^{35}S]methionine/dish. Alpha 2-macroglobulin was immunoprecipitated from comparable amounts of TCA-precipitable radioactivity from the culture media, subjected to SDS-PAGE and autoradiography as detailed in section 2. Preabsorption of unspecifically bound material by non-immune control serum is shown in (A), lane 1. Displacement of radiolabeled alpha 2-macroglobulin by addition of $50 \mu\text{g}$ unlabeled alpha 2-macroglobulin to the medium prior to immunoprecipitation is shown in (A), lane 6 (compared to lane 5).

[14,28]. By balancing the amazing variety of endogenous proteases, protease inhibitors control important proteolytic functions such as coagulation and fibrinolysis, activation of the complement system and tissue breakdown. In the central nervous system, neurite outgrowth has been found to depend on a precarious balance between proteases and protease inhibitors at and around the neuritic growth cones [3]. IL-6 can be produced in the central nervous system by microglia and astrocytes under inflammatory conditions [31]. Therefore, it could be that IL-6 induced a2M synthesis may affect neuronal plasticity under inflammatory conditions.

Impaired APP secretion in the presence of a2M could point to a potential inhibition of the proteolytic cleavage which is necessary for the release of the secretory part of the APP molecule. It has previously been shown that the intracellularly synthesized mature APP protein is inserted into the cell membrane [10]

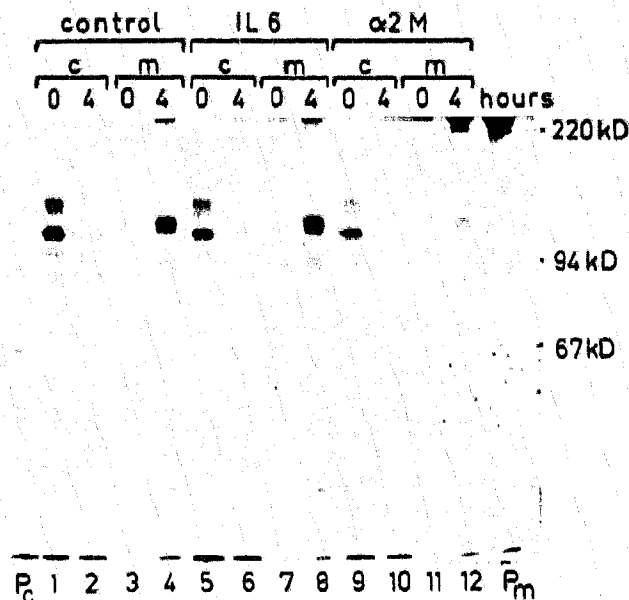


Fig. 3. βA4 Amyloid precursor protein (APP) synthesis and secretion by human neuronal cells cultured in the absence (lanes 1-4) and presence of interleukin-6 (lanes 5-8) or alpha 2-macroglobulin (lanes 9-12). Human SH-SY5Y neuroblastoma cells (10^6 cells/40 mm dish) were cultivated for 5 days as described in section 2. For an additional 72 h, cells were either incubated as before (lanes 1-4), or in the presence of 200 units/hr interleukin-6 (lanes 5-8), or the presence of alpha 2-macroglobulin in a final concentration of 1 mg/ml (lanes 9-12). For metabolic pulse-labelling, media were replaced by serum- and methionine-free RPMI 1640 medium containing $200 \mu\text{Ci}$ [^{35}S]methionine/dish. After 20 min, cells were either harvested immediately (lanes 1,3,5,7,9,11), or radiolabelled methionine was chased by an excess of unlabeled methionine (2 mM final concentration) for 4 h (lanes 2,4,6,8,10,12). Cultures were harvested by separating media from cells. βAPP was immunoprecipitated from one third of the cell homogenates (lanes 1,2,5,8,10) and from total culture media (lanes 3,4,7,8,11,12), subjected to SDS-PAGE and autoradiography as described in section 2. Preabsorption of unspecifically bound material by non-immune control serum is shown in Pc (for cell homogenates) and Pm (for media). Total de-novo synthesized intracellular proteins (determined as TCA-precipitable cpm) at the end of the pulse labeling (i.e. 0 h of chase) was 3.17×10^6 cpm in control cells; 3.09×10^6 cpm in IL-6-treated cells; and 2.22×10^6 cpm in a2M-treated cells. Total de-novo synthesized and secreted proteins after 4 h chase (determined as above) amounted to 1.11×10^6 cpm (Co), 1.19×10^6 cpm (IL-6), and 1.13×10^6 cpm (a2M). If, after standardization with respect to total protein synthesis/secretion, intracellular (0 h chase) APP and secreted APP (4 h chase) in a2M-treated cultures was compared to controls, then intracellular a2M was reduced by 22% and secreted a2M by 83%.

followed by cleavage of APP resulting in secretion of the large N-terminal APP portion. This cleavage happens inside the βA4 sequence of the APP protein, C-terminal to residue 16 of the βA4 part of APP [12,13]. Thus, unaltered APP cleavage by the still unknown protease (called secretase) prevents the formation of the βA4 protein which is found as a main component of the cortical senile plaques in Alzheimer's disease [32]. It must be assumed that inhibition of the still unknown protease/secretase is part of the pathophysiological

events in Alzheimer's disease. Further studies are under way to exactly relate the antiproteolytic potential of a2M to its effects on APP processing (L. Sottrup-Jensen, J. Bauer, in preparation).

Our hypothesis that IL-6-induced a2M synthesis may be part of Alzheimer's disease pathophysiology has been supported by our recent finding that senile plaques in brains of Alzheimer's disease patients strongly stain for both IL-6 and a2M (B. Volk and J. Bauer, submitted for publication).

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