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Macrophage-mediated myelin-related mitogenic factor for cultured Schwann cells

(Wallerian degeneration/mitogen/lysosomal processing)

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ABSTRACT **Conditioned medium from cultured peritoneal macrophages that have phagocytosed a myelin membrane fraction is mitogenic for cultured Schwann cells. Production of the mitogenic supernatant was time- and dose-dependent with a maximal Schwann cell-proliferative response from supernatants after 48-hr incubation of cultured macrophages with myelin-enriched fraction (200 μg of protein per ml). The response was specific for myelin membrane: supernatants derived from macrophages incubated with axolemma, liver microsomes, polystyrene beads, or lipopolysaccharide were not mitogenic. Lysosomal processing of the myelin membrane was necessary for the production of the mitogenic factor, which was shown to be heat labile and trypsin sensitive. There was no species specificity because myelin membranes isolated from the central and peripheral nervous systems of rat, bovine, and human were equally potent in eliciting mitogenic supernatant. However, supernatants derived from central nervous system myelin membranes were two to three times more mitogenic than those obtained from peripheral nervous system fractions of the same species. Previous observations that myelin is mitogenic for cultured Schwann cells may, in part, involve the intermediate processing of myelin by macrophages that are present in Schwann cell cultures. These results suggest that macrophages play a crucial role in Schwann cell proliferation during Wallerian degeneration.**

The source of the mitogenic signal for Schwann cell division during Wallerian degeneration is not clear. Removal of myelin sheaths accompanying Wallerian degeneration has been ascribed to Schwann cells (1, 2) as well as macrophages (3–5). Beuche and Friede (5) observed that Wallerian degeneration proceeding without nonresident phagocytic cells (macrophages) showed no Schwann cell proliferation and no active intracellular digestion of myelin—implying that myelin membranes were removed solely by macrophages after nerve degeneration. This observation also suggests that Schwann cell proliferation is related to myelin removal by macrophages. Salzer and Bunge (6) using dorsal root ganglion explant cultures showed that only myelin-related Schwann cells proliferate after axotomy. In contrast, however, *in vivo* studies by Pellegrino *et al.* (7) on cat peripheral nerve suggest that Schwann cell mitosis during nerve degeneration is due to loss of axonal contact, and observations by Crang and Blakemore (3) on explant cultures of cat sciatic nerve suggest that Schwann cell division during Wallerian degeneration occurs without macrophages.

We are investigating Schwann cell proliferation in response to membrane mitogens using an *in vitro* system. Cultured Schwann cells prepared by the method of Brockes *et al.* (8) can be stimulated to proliferate by an axolemma-enriched fraction (AEF) and a myelin-enriched fraction (MEF) (9). The presence of macrophages in our Schwann cell cultures and the possible involvement of macrophages in removal of myelin debris during Wallerian degeneration (10) prompted us to study the role of macrophages in mediating mitogenicity of myelin for cultured Schwann cells. Using rat peritoneal macrophages, we have shown that macrophages stimulated with MEF produce a soluble factor(s) mitogenic for cultured Schwann cells. Production of the soluble mitogenic factor shows a time- and dose-dependent response. Other membrane fractions or nonspecific agents do not stimulate macrophages to produce a mitogenic-conditioned medium. The myelin membrane undergoes lysosomal processing in the macrophage before mitogenic factor is produced. Sensitivity of the mitogenic supernatant to heat and trypsin treatment suggests that the mitogenic factor is a polypeptide.

MATERIALS AND METHODS

Preparation of Schwann Cells. Schwann cell cultures were prepared according to the method of Brockes *et al.* (8). Briefly, sciatic nerves were excised from 2- to 3-day-old Sprague–Dawley rats. Pooled nerves were enzymatically dissociated using 0.25% collagenase and 0.25% trypsin. The mixture was triturated using a Pasteur pipette and filtered through a 209-μm Nitex (Tetko) filter. Cells were collected by centrifugation and resuspended in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (DMEM/FCS) and plated on 100-mm glass dishes at a density of 4–6 $\times 10^6$ cells per dish. To reduce fibroblast contamination, 1 day after initial plating the cultures were treated with an antimitotic agent, cytosine arabinoside (final concentration of 10^{-5} M), for 2 days. One day after removal of cytosine arabinoside cells were plated onto glass coverslips in 24-well culture plates at a density of 40,000 cells per well for the autoradiographic assay.

Macrophage Cultures. Adult Sprague–Dawley rats were injected i.p. with 10 ml of 3% (wt/vol) thioglycollate medium (Difco). After 3–4 days peritoneal macrophages were harvested by peritoneal lavage using physiological saline (11) and plated in DMEM/FCS at a density of 1×10^6 cells per 2 ml in 6-well plastic plates. The next day macrophage cultures were washed with saline and stimulated in DMEM/FCS with membrane fractions or other agents for 48 hr or the specified time period (8, 16, 24, 32, or 40 hr). Control conditioned media were supernatants from resting macrophage cultures incubated for the appropriate time periods in DMEM/FCS alone.

Abbreviations: AEF, axolemma-enriched fraction; MEF, myelin-enriched fraction; C, control conditioned medium; CM, myelin-stimulated conditioned medium; PNS, peripheral nervous system; CNS, central nervous system; IL-1, interleukin 1; DMEM/FCS, Dulbecco's modified Eagle's medium plus fetal calf serum; hmu, half-maximal unit of biological activity.

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After incubation, the macrophage-conditioned media were filtered through 0.22-μm filter (Millipore) to remove membranes, and the media were stored at −80°C.

Autoradiographic Assay for Schwann Cell Proliferation. Control and macrophage-conditioned media were diluted 1:1 with DMEM/FCS and added to Schwann cells on coverslips. Tritiated thymidine (3 μCi/ml) (New England Nuclear, 18.2 Ci/mmol; 1 Ci = 37 GBq) were added per well, and cultures were incubated for 48 hr. After incubation, cells were washed and processed for autoradiography as described (12). A minimum of 800–1000 cells per coverslip was counted, and a labeling index was determined by a semiautomated image analysis system (13).

Interleukin-1 (IL-1) (Collaborative Research, Bedford, MA) was reconstituted in sterile distilled water to give a stock solution of 510 hmu/ml (half-maximal unit of biological activity as described by the supplier). IL-1 was added to Schwann cell cultures to final concentrations of 0.25, 2.5, and 25 hmu/ml. Tritiated thymidine (3 μCi/ml) was added per well, and the cultures were incubated for 48 hr. After incubation, cultures were washed and processed for autoradiography. Control Schwann cell cultures received DMEM/FCS alone.

Preparation of AEF and MEF. AEF and MEF from central nervous system (CNS) (14) and peripheral nervous system (PNS) (15) were prepared as described previously. Membrane fractions were stored at −80°C in small aliquots in sterile saline I (138 mM NaCl/5.4 mM KCl/1.1 mM Na_2HPO_4/1.1 mM KH_2PO_4/22 mM dextrose, pH 7.0).

Esterase Staining of Macrophage Cultures. Peritoneal macrophage cultures were stained for nonspecific esterase activity using α-naphthyl acetate as a substrate by the method of Yam *et al.* (16).

Electron Microscopy. Cultured peritoneal macrophages that had been treated with MEF for 24 or 48 hr were fixed and processed for electron microscopy as described (17).

RESULTS

Phagocytosis of MEF by Peritoneal Macrophages. The identity of peritoneal macrophages was confirmed morphologically by light and electron microscopy and histochemically by esterase staining. Cultured peritoneal macrophages were tested for their ability to phagocytose MEF. Fig. 1 shows an electron micrograph of peritoneal macrophage incubated with a MEF for 24 hr. Cells contained numerous myelin phagosomes in various stages of degradation; the cytoplasm also contained large numbers of lipid-filled vacuoles.

Time Course for the Production of Mitogenic Supernatants from MEF-Stimulated Macrophages. Peritoneal macrophage cultures were incubated either with medium alone or with rat CNS MEF (200 μg of protein per ml) for varying periods of time ranging from 8–48 hr. Supernatants were collected and added to Schwann cell cultures after diluting 1:1 with DMEM/FCS. Fig. 2 shows that supernatants from macrophage cultures incubated with myelin membranes for increasing periods of time were increasingly mitogenic to Schwann cell cultures. Maximal labeling percentage of 6.9% (±1.0%) was obtained with 48-hr macrophage-conditioned medium. Cultures receiving control conditioned media contained only 0.4% (±0.1%) labeled cells. We found that myelin-stimulated macrophage-conditioned supernatants after 72-hr incubation decreased 30% from the maximal mitogenic response of 48 hr.

Dose–Response of Production of Mitogenic Supernatants by Macrophages After Addition of MEF and AEF. Fig. 3 shows Schwann cell labeling in response to macrophage-conditioned media from adding increased dosages of either rat CNS MEF or AEF to cultured macrophages for 48 hr. Addition of increased amounts of MEF to cultured macrophages for 48 hr produced supernatants with dose-dependent mitogenicity increases for Schwann cells. Supernatants from added AEF produced stimulation of Schwann cell cultures comparable to that obtained by control conditioned medium.

Production of Mitogenic Supernatant by Macrophages in Response to Nonspecific Stimuli. The myelin specificity of the macrophage-mediated mitogenic factor for cultured Schwann cells was studied using membrane fractions other than myelin, AEF or rat liver microsomes, and other agents such as polystyrene beads (1 μm diameter) and lipopolysaccharide from *Escherichia coli* and *Salmonella* (18, 19). Results shown in Table 1 indicate that medium conditioned by the addition of any of these agents to cultured macrophages for 48 hr did not produce mitogenic supernatant for Schwann cells.

Requirement for Lysosomal Processing of Myelin Membrane for the Production of the Mitogenic Factor(s). An

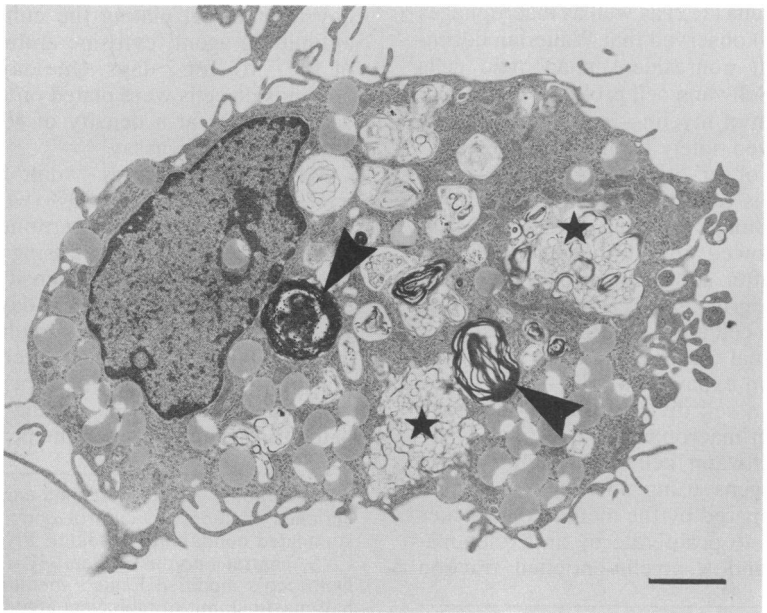

FIG. 1. An electron micrograph of a peritoneal macrophage incubated with MEF for 24 hr. Arrowheads indicate phagocytosed myelin in a relatively compact state. Stars represent myelin in an advanced state of degradation. Numerous lipid-filled vacuoles are also present in the macrophage cytoplasm. Bar = 2.0 μm.

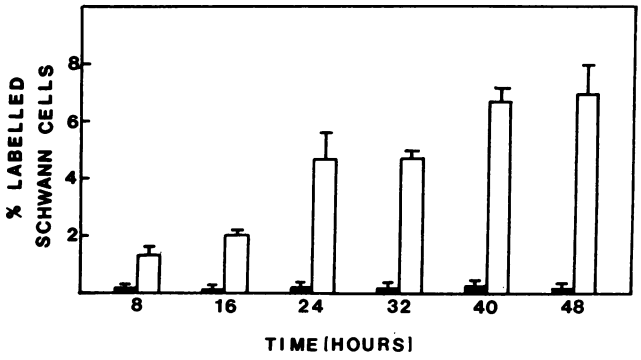

FIG. 2. Time course for production of mitogenic supernatant for cultured Schwann cells stimulated with MEF. Macrophage cultures were incubated either alone (black bars) or with MEF (200 μg of protein per ml) (white bars) from 8–48 hr. Supernatants after incubation were added to cultured Schwann cells with tritiated thymidine for 48 hr. Cells were washed and processed for autoradiography. Values are reported as mean ± SD for $n = 3$.

increase in lysosomal enzyme activity has been seen in nerves undergoing degeneration (2). We have previously shown that the mitogenicity of myelin membrane to cultured Schwann cells is reduced in the presence of ammonium chloride, an inhibitor of lysosomal activity (9). From these observations we tested the effect of ammonium chloride (7 mM) on the production of the mitogenic factor(s) by myelin-stimulated macrophages. Fig. 4 shows that incubation of macrophages with myelin with ammonium chloride reduced mitogenicity of the conditioned medium by ≈75%. Similar results were obtained using L-methionine methyl ester, which also inhibits lysosomal activity (20) but does not increase intralysosomal pH (data not shown). The sensitivity of macrophage production of the mitogenic factor to agents inhibiting lysosomal activity suggests that lysosomal processing of the myelin membrane is necessary for mitogenic factor production.

Biochemical Characterization of the Myelin-Stimulated Macrophage-Conditioned Medium. Table 2 shows the effects of heat and trypsin treatments on the mitogenic activity of myelin-stimulated macrophage-conditioned medium. Sensitivity to heat and trypsin treatment, as seen from the proliferative response, suggests that the mitogenic factor(s) in myelin-treated supernatants is a polypeptide. However, mitogenic activity in the conditioned medium was resistant

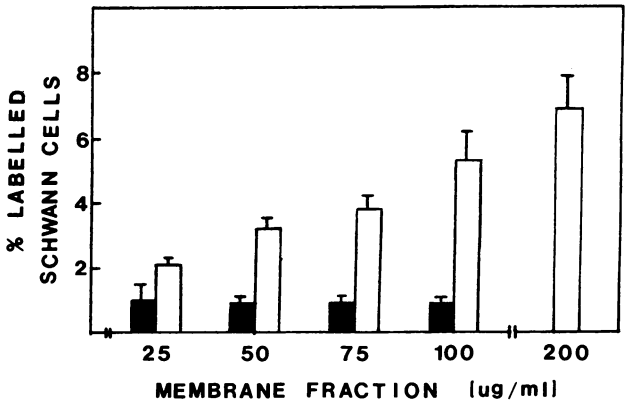

FIG. 3. Autoradiographic analysis of cultured Schwann cells stimulated with macrophage-conditioned medium from addition of increasing amounts of either AEF (black bars) or MEF (white bars) to macrophage cultures for 48 hr. Schwann cells treated with control supernatant from macrophage-conditioned medium without membranes had a labeling index of 0.9% (±0.2%). Values are reported as mean ± SD for $n = 3$.

Table 1. Effect of macrophage culture additives on macrophage-conditioned media-induced Schwann cell proliferation

Additives	Schwann cell labeling, %
None	0.6
Rat CNS myelin membrane (50 μg/ml)	3.2
Rat liver microsomes (50 μg/ml)	0.9
Lipopolysaccharide	
E. coli (10 μg/ml)	0.3
Salmonella (10 μg/ml)	0.3
Polystyrene beads	
(1-μm diameter) (250 μg/ml)	0.4

Macrophage cultures were treated with indicated additives at specified concentrations for 48 hr. Supernatants were harvested and added to Schwann cell cultures to obtain a labeling index. Values reported are an average of duplicate determinations.

to 2-mercaptoethanol treatment (data not shown), which implies the activity may differ from the previously described platelet-derived growth factor-like activity produced by macrophages (which is sensitive to this treatment) (21). The inability of macrophages to produce a mitogenic supernatant for cultured Schwann cells in response to lipopolysaccharide stimulation (Table 1) suggests that the mitogenic factor may not be IL-1 (18, 19). We have also tested commercially available IL-1 for its ability to stimulate Schwann cell proliferation at concentrations of 0.25 hmu/ml, 2.5 hmu/ml, and 25 hmu/ml. IL-1 was not mitogenic to cultured Schwann cells: the labeling percentage of the cultures was 1.2%, 0.9%, and 0.8%, respectively, in response to the tested concentrations. Control cultures gave a labeling index of 0.8%. The mitogenic factor present in myelin-treated supernatants appears unstable. A 25% loss of activity was seen after one freeze–thaw cycle. However, the mitogenic factor is stable up to 2 months if stored at −80°C.

Production of the Mitogenic Factor by Macrophages Stimulated with Myelin Membrane from Different Species. The species specificity of myelin in mitogenic factor production by macrophages was tested using MEF obtained from rat, bovine, and human CNS and PNS. Fig. 5 shows the Schwann cell labeling in response to conditioned media obtained by adding rat, bovine, and human CNS and PNS myelin (75 μg of protein per ml) to cultured macrophages.

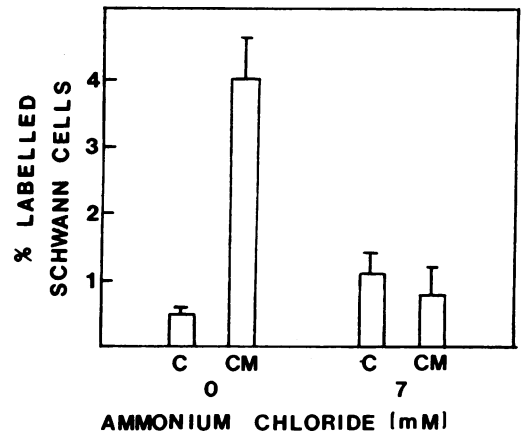

FIG. 4. Effect of ammonium chloride on the production of the mitogenic supernatant. Macrophage cultures were incubated either in the presence (CM) or absence (C) of MEF (25 μg of protein per ml) for 48 hr with (right two bars) or without (left two bars) 7 mM ammonium chloride. Supernatants were added to Schwann cell cultures and incubated with tritiated thymidine for 48 hr. Cells were washed and processed for autoradiography. Values are reported as mean ± SD for $n = 3$.

Table 2. Biochemical characteristics of myelin-stimulated macrophage-conditioned medium

Treatment	Type of medium	Labeling, %	Proliferative response, % ratio*
None	C	0.6 (0.2)	
	CM	3.0 (0.4)	4.5
Heat (100°C, 10 min)	C	0.5 (0.1)	
	CM	0.5 (0.2)	1.0
None	C	0.4 (0.0)	
	CM	2.7 (0.2)	6.7
Trypsin† (0.05%, 30 min, 37°C)	C	0.5 (0.1)	
	CM	0.4 (0.2)	1.2

Myelin-stimulated macrophage-conditioned media (CM) and control (no myelin) conditioned media (C) were exposed to the indicated treatments. Supernatants were added to Schwann cell cultures to obtain a labeling index. Values are reported as average ± SD for $n = 3$ in nontreated condition. Treatment values are an average of two determinations, and numbers in parentheses indicate ranges of variation.

*Proliferative response equals the percent labeling with CM/percent labeling with C.

†Trypsin treatment was followed by inactivation of trypsin with soybean trypsin inhibitor.

The data indicate that the ability of peritoneal macrophages to release mitogenic factor is not species restricted. CNS fractions from different species were equally potent in eliciting the mitogenic supernatant; PNS fractions were also equally potent. However, conditioned media obtained by the addition of CNS myelin were two to three times more mitogenic than those derived from PNS myelin from all species.

DISCUSSION

In addition to axonal loss, Wallerian degeneration is accompanied by the breakdown and removal of myelin sheaths and proliferation of Schwann cells (22, 23). Removal of myelin debris after axonal degeneration has, in part, been attributed to macrophages infiltrating the site of injury (3–5). The nature of the mitogenic signal for Schwann cell mitosis at this stage is yet unknown. Studies by Beuche and Friede (5) with Millipore diffusion chambers implied a connection between Schwann cell division and infiltration by macrophages. We have previously shown that myelin is mitogenic for cultured Schwann cells (9). These observations taken in conjunction with the fact that we have identified macrophages in our Schwann cell culture (10) strongly suggested that macrophages play a role in mediating the mitogenicity of the myelin membrane to cultured Schwann cells. We have demonstrated that cultured peritoneal macrophages, when presented with a MEF, produce supernatants that are mitogenic to cultured Schwann cells. This response is specific for myelin membrane only (Table 1), although this response is not species restricted (Fig. 5). That the mitogenic activity of the conditioned medium from PNS myelin addition to macrophages is lower than that from CNS myelin addition may mean that any mitogenic factor inherent to the myelin membrane is present in greater amounts in CNS myelin than in PNS myelin. Preliminary biochemical characterization of the mitogenic factor in the myelin-conditioned medium implies the factor is a polypeptide (Table 2).

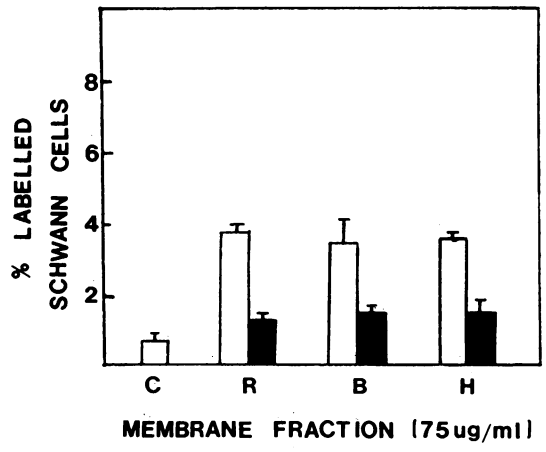

FIG. 5. Autoradiographic analysis of cultured Schwann cells stimulated with macrophage-conditioned medium from addition of either CNS (white bars) or PNS (black bars) MEF (75 μg of protein per ml) from rat (R), bovine (B), and human (H). C represents the labeling index of Schwann cell cultures in response to incubation with supernatants from control macrophage cultures. Error bars represent the range of values for duplicate determinations.

In Fig. 3 we show that only myelin- and not axolemma-stimulated macrophage-conditioned medium is mitogenic for cultured Schwann cells. We have previously shown that both AEF and MEF are mitogenic when added directly to cultured Schwann cells (9). That MEF and not AEF stimulates macrophages to produce mitogenic supernatants for cultured Schwann cells supports our previous findings that mitogenic signals in the two membrane fractions are distinct. The mitogenic signal for Schwann cell division during development (before the formation of myelin sheath) has been attributed to the growing axon (24). However, the signal for Schwann cell proliferation in Wallerian degeneration is not yet known. Possibly the two mitogenic signals we observe for cultured Schwann cells from the membrane fractions represent the two signals for Schwann cell division *in vivo*. The mitogenic signal in AEF may relate to the developmental phase of Schwann cell proliferation and be mediated by cell contact, whereas Schwann cell mitosis during Wallerian degeneration may occur in response to a soluble mitogenic factor released by the infiltrating macrophages after phagocytosis of the myelin debris.

From our observations we propose a general model for the macrophage-mediated mitogenicity of MEF to cultured Schwann cells. We have shown that myelin membrane requires lysosomal processing within the macrophage for the release of the factor (Fig. 4). At least two mechanisms may be responsible for mitogenic factor production. The factor may be an integral part of the myelin membrane, in which case macrophages act as digesting machinery and release the factor after processing the membrane. Macrophages are known to process foreign material and release a part of it as seen during antigen presentation by macrophages in immunological reactions (25).

On the other hand, the macrophages may actually synthesize a growth factor in response to some specific component present in myelin membrane. Macrophages secrete a number of biologically active substances, such as growth factors, enzymes, and prostaglandins (26, 27). The growth-promoting activity of the macrophages and their conditioned media has been demonstrated for several different cell types, such as fibroblasts, vascular smooth muscle cells, and vascular endothelial cells (28–30). Products secreted by macrophages have been implicated in the proliferation of these cells following pathophysiological phenomena such as neovascularization (31–33), wound healing (34), development of an atherosclerotic plaque (35), and proliferative glomerulonephritis (36). In all these cases, proliferation of the various cell types was in close association with monocytic infiltration at the site of injury.

During Wallerian degeneration, the infiltration of macrophages for the removal of myelin debris and concomitant Schwann cell proliferation, possibly in response to a soluble

mitogenic factor released by the macrophages, may represent another example of the critical role of macrophages in a pathophysiological process.

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