Green Tea Polyphenols Block Endotoxin-Induced Tumor Necrosis Factor-Production and Lethality in a Murine Model

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ABSTRACT Green tea polyphenols are potent antioxidants. They have both anti-cancer and anti-inflammatory effects. However, their mechanisms of actions remain unclear. In inflammation, tumor necrosis factor-α (TNFα) plays a pivotal role. NF-κB, an oxidative stress-sensitive nuclear transcription factor, controls the expression of many genes including the TNFα gene. We postulated that green tea polyphenols regulate TNFα gene expression by modulating NF-κB activation through their antioxidant properties. In the macrophage cell line, RAW264.7, (-)-epigallocatechin gallate (EGCG), the major green tea polyphenol, decreased lipopolysaccharide (LPS)-induced TNFα production in a dose-dependent fashion (50% inhibition at 100 mmol/L). EGCG also inhibited LPS-induced TNFα mRNA expression and nuclear NF-κB–binding activity in RAW264.7 cells (30–40% inhibition at 100 mmol/L). Similarly, EGCG inhibited LPS-induced TNFα production in elicited mouse peritoneal macrophages. In male BALB/c mice, green tea polyphenols (given by oral gavage 2 h prior to an i.p. injection of 40 mg LPS/kg body wt) decreased LPS-induced TNFα production in serum in a dose-responsive fashion. At a dose of 0.5 g green tea polyphenols/kg body wt, serum TNFα was reduced by 80% of control. Moreover, 0.5 g green tea polyphenols/kg body wt completely inhibited LPS-induced lethality in male BALB/c mice. We conclude that the anti-inflammatory mechanism of green tea polyphenols is mediated at least in part through down-regulation of TNFα gene expression by blocking NF-κB activation. These findings suggest that green tea polyphenols may be effective therapy for a variety of inflammatory processes.


KEY WORDS: mice • polyphenols • tea • endotoxin • tumor necrosis factor

Cytokines serve as intercellular signals that recruit cells and modulate cell function. Cytokines produced predominantly by activated macrophages and lymphocytes mediate many inflammatory processes (Brennan and Feldman 1996). These proinflammatory cytokines include interleukin-1 (IL-1)4, tumor necrosis factor-α (TNFα) and chemokines (e.g. interleukin-8 [IL-8], macrophage chemotactic and activating factor [MCAF]). Though all of these cytokines play important roles in the evolving inflammatory response, TNFα appears to be a critical mediator of the inflammatory cascade. Numerous studies show that TNFα rises rapidly following acute trauma/inflammation/infection and that blocking TNFα activity reduces injury (Beutler and Cerami 1986, Machleidt et al. 1996, Pfeffer et al. 1993, Tracey et al. 1986 and 1987).

The increase in cytokines following stimulation occurs as a result of gene expression and de novo synthesis. Nuclear factor-κB (NF-κB), an oxidative stress sensitive transcription factor, controls the expression of a wide variety of genes active in inflammation that include cytokines (e.g., IL-1, TNFα, IL-8), enzymes (inducible nitric oxide synthase [iNOS], IL-1β), nuclear factor-κB (NF-κB) and transcription factor-κB (κB) in the promoter region and induces gene expression. Known anti-inflammatory agents (e.g., sodium salicylate, dexamethasone), antioxidants and proteasome inhibitors block NF-κB activation (Beaufort and Hiscott 1996). These observations suggest that NF-κB is a suitable target to prevent or reduce an inflammatory response.

There is increasing interest in the role of nutrients in health and disease. One such nutrient is tea. Epidemiological studies suggest that regular tea consumption reduces the risk of...
cancer (Katiyar and Mukhtar 1996, Stoner and Mukhtar 1995). Although tea consists of several components, interest has focused primarily on polyphenols, especially those found in green tea. Assuming the consumption of three cups (~300 mL) of tea daily, roughly 240–320 mg of polyphenols are provided. The green tea polyphenols include (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epicatechin (EC). Of these, EGCG accounts for >40% of the total (Salah et al. 1993). Following consumption, the polyphenols remain predominantly in their conjugated forms and are primarily excreted intact in the urine (Lee et al. 1995).

Polyphenols have potent antioxidant properties including the scavenging of oxygen radicals and lipid radicals (Salah et al. 1995). There is increasing evidence that green tea polyphenols have anti-inflammatory effects, possibly mediated through their antioxidant properties. For instance, EGCG inhibits kDa acid-induced TNFα production and gene expression in BALB/3T3 cells (Suganuma et al. 1996), and EGCG inhibits lipopolysaccharide (LPS)-induced NO production and iNOS gene expression in isolated peritoneal macrophages by decreasing NF-kB activation (Lin and Lin 1997). These preliminary findings suggest that green tea polyphenols may have utility as novel nutrient therapy in inflammatory processes.

In this study, we further examined the anti-inflammatory effects of green tea polyphenols. We examined the effects of EGCG on LPS-mediated TNFα mRNA expression and protein production in both the macrophage cell line, RAW264.7, and in elicited murine peritoneal macrophages. We also examined the effects of orally administered green tea polyphenols on TNFα production and survival in a murine model of LPS-mediated lethality.

MATERIALS AND METHODS

Materials. Extracted green tea polyphenols (95% pure) were purchased from LKT Laboratories, Inc. (St. Paul, MN). (-)-Epigallocatechin-3-gallate (EGCG) (98% pure), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), lipopolysaccharide (LPS, from E. coli, Serotype 0111:B4) and other chemical reagents were purchased from Sigma (St. Louis, MO). Bio-gel beads were purchased from Bio-Rad Laboratories (Hercules, CA). All cell culture supplies were purchased from Gibco BRL (Grand Island, NY).

Animals. Male BALB/c mice (6–8 wk old) approximately 20–25 g in body weight were obtained from Harlan Sprague Dawley (Indianapolis, IN). The mice were housed at the Veterans Administration Medical Center in Lexington, KY, in standard steel cages with free access to unpurified diet (Harlan Teklad Laboratory diet #8604, Madison, WI) and water. This study was approved by and performed in accordance with the guidelines for the care and use of laboratory animals at the Veterans Medical Center in Lexington, KY.

Isolation and treatment of peritoneal macrophages. Mouse peritoneal macrophages were isolated according to the method described previously (Fauve et al. 1983). Each BALB/c mouse was intraperitoneally injected with 1 mL of sterile phosphate-buffered saline (PBS) containing 10 g Bio-gel beads/L. After 4 d, 10 mL of sterile PBS was injected into the peritoneum and withdrawn. The collected cells then were washed once with PBS, once with serum-free Opti-MEM1 and resuspended in Opti-MEM1. The cells (2 × 10⁶ cells/well) were plated onto 96-well plates and cultured for 45 min to allow adherence. The plates were then washed three times with sterile PBS to remove the nonadherent cells. The resulting adherent cell population consisted of >95% macrophages as determined by detecting nonspecific esterase activity. For measuring TNFα protein, cells were pre-treated with 50 mmol EGCG/L for 2 h, and then stimulated with 10 mg LPS/L overnight in serum-free Opti-MEM1 medium at 37°C in an atmosphere of 10% CO₂ and 95% relative humidity. The conditioned culture supernatants were collected and stored (~70°C).

Cell culture. The mouse macrophage cell line, RAW264.7 was purchased from ATCC (Rockville, MD). RAW264.7 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) endotoxin-free fetal calf serum, 2 mmol glutamine/L and 1,000,000 IU/L of penicillin/streptomycin at 37°C in an atmosphere of 10% CO₂ and 95% relative humidity. Passage consisted of incubating cells in sterile PBS containing 5 mmol EDTA/L for 15 min followed by pelleting the cells and resuspending the cells in fresh medium. For the TNFα immunossay experiments, cells (2 × 10⁶ cells/well) were plated onto 96-well plates and exposed to various concentrations of EGCG (0–200 mmol/L) for 2 h by followed the addition of 10 mg LPS/L for 6 h. The culture supernatants then were collected and stored at ~70°C.

MTT assay. The MTT assay was used to assess the effects of EGCG on cell viability (Hansen et al. 1989). The assay relies on production of a colored formazan by the action of mitochondrially encoded enzymes on MTT. The elicited peritoneal macrophages and RAW264.7 were plated as described above. Following an overnight incubation with various concentrations of EGCG (0–800 mmol/L), the cells were washed twice with PBS and then incubated in fresh medium containing 1 g MTT/L at 37°C for 1 h. Then, an equal volume of lysis buffer (pH = 4.7) containing 200 g SDS/L and of 50% (v/v) of N,N-dimethylformamide was added to the cultures, which were incubated overnight. The degree of formazan produced was measured by the spectrophotometer (absorbance at 570 nm).

TNFα Immunosay. TNFα protein was detected in cell culture supernatants and mouse serum using a mouse TNFα ELISA kit from Endogen, Inc. (Woburn, MA).

Northern blot analysis. Total RNA was isolated from RAW264.7 cells using an acid guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi 1987). The quality of the RNA was confirmed by measuring the O.D.260/280 ratio. Total RNA was then visualized by autoradiography. As internal controls, the same membrane was stripped and rehybridized with ²P-labeled oligonucleotides specific for mouse TNFα mRNA (Clontech Laboratories, Palo Alto, CA). The TNFα mRNA was then visualized by autoradiography. As internal controls, the same membrane was stripped and rehybridized with ³²P-labeled oligonucleotides specific for mouse glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA (Clontech Laboratories). Densitometry was analyzed using a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA) with ImageQuant V1.1 (Molecular Dynamics).

Nuclear extraction. Nuclear extracts were isolated by a modified method initially described by Dignam et al. (1983). RAW264.7 cells (1 x10⁶ cells /plate) were seeded onto 60 mm culture plates and incubated as described above for 2 d. The cultures were then treated with or without 100 nmol EGCG/L for 2 h, followed by exposure to 10 mg LPS/L for various periods of time. The cells then were washed twice with ice-cold PBS followed by incubation on ice for 15 min with 0.2 mL of ice-cold lysis buffer [10 mmol HEPES/L (pH=7.9), 1.5 mmol MgCl₂/L, 10 mmol KCl/L, 0.5 mmol dithiothreitol (DTT)/L, 0.5 mmol phenylmethylsulfonyl fluoride/L (PMSF), 0.1% (v/v) Igepal CA-630, 1 mg leupeptin/L, 1 mg pepstatin/L and 1 mg leucine thiol/L]. Cells were then scraped and collected in 1.5-mL polypropylene tubes and placed on ice for an additional 30 min. The homogenates then underwent centrifugation at 4°C at 1,200 × g for 10 min. The resulting pellets were washed once with 0.5 mL of ice-cold lysis buffer and incubated on ice for 1 h with 80 μL of nuclear extraction buffer [20 mmol HEPES/L (pH=7.9), 25% (v/v) glycerol, 0.52 mol NaCl/L, 1.5 mmol MgCl₂/L, 0.1 mmol EDTA/L, 0.5 mmol DTT/L, 0.5 mmol PMSF/L, 0.1% Igepal CA-630, 1 mg leupeptin/L, 1 mg pepstatin/L and 1 mg leucine thiol/L]. The resulting homogenates underwent centrifugation at 4°C at 110,000 × g for 15 min. The supernatants were collected and stored at −70°C until use.
Electrophoretic mobility shift assay for NF-κB. NF-κB, which binds to -κB enhancer elements on DNA, was detected in nuclear extracts by an Electrophoretic mobility shift assay (EMSA) as described by Sen and Baltimore (1986). 32P-end-labeled double-stranded DNA probes (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) containing NF-κB consensus sequences were prepared. Nuclear extract containing 8 mg of total proteins was incubated with end-labeled probes for 20 min at room temperature in the presence of 4 mg poly(dl-ic)/L (Pharmacia Biotech, Piscataway, NJ). Complexed and uncomplexed DNA were then resolved by electrophoresis on a 5% low ionic strength nondenaturing polyacrylamide gel and visualized by autoradiography. Supershift experiments were performed by incubating nuclear extracts with antibodies against p65 or p50 for 30 min before adding 32P-labeled probes. Competition experiments were performed by adding unlabeled NF-κB consensus as a specific competitor or by adding sonicated salmon sperm DNA (GIBCO BRL, Grand Island, NY) as a nonspecific competitor.

Measurement of polyphenols in serum. Polyphenols were detected in serum by the Prussian blue assay initially described by Price and Butler (1977) with slight modification. Serum proteins were removed by adding 100 g trichloroacetic acid/L and centrifuged at 10,000 × g for 5 min. The resulting supernatant was diluted 100-fold, and 50 mL of 100 mmol FeCl3/L (in 0.1 mol HCI/L) and 4 mL of 10 mmol K3Fe(CN)6/L were added to each mL of the diluted solution. After brief vortexing, the mixture was incubated at room temperature for 20 min, and the absorbance was determined at 720 nm. To calculate the polyphenol concentration in samples, a standard curve was made by using solutions containing known concentrations of green tea polyphenols.

Statistical analysis. Where indicated, results are expressed as means ± sd. Statistical significance of the difference between two independent groups was determined by using a two-tail Student’s t-test. P < 0.05 was considered to be significant.

RESULTS

EGCG inhibits LPS-induced TNFα gene expression and protein production in RAW264.7 cells by blocking NF-κB activation. To examine the effect of green tea polyphenols on cytokine production, we first examined the effects of EGCG on TNFα production in the murine macrophage cell line, RAW264.7. The cells were pretreated with 0–200 mmol EGCG/L for 2 h and then exposed to (10 mg LPS/L for 6 h. The conditioned culture supernatants were collected and TNFα protein was assayed by ELISA. EGCG induced a significant decrease in TNFα protein levels in a dose-response fashion (Fig. 1). In the presence of 100 mmol EGCG/L, LPS-mediated TNFα protein levels decreased by nearly 50% (P < 0.01 vs. control). Interestingly, EGCG alone caused a modest increase in TNFα protein.

We next performed Northern blot analysis to determine whether this reduction in TNFα protein was associated with decreased gene transcription. Northern blot analysis showed that EGCG decreased LPS-mediated TNFα mRNA expression in a dose dependent fashion (Fig. 2A). Similar to the ELISA data, 100 mmol of EGCG/L decreased LPS-mediated TNFα mRNA expression by nearly 45% (Fig. 2B). These data show that EGCG decreases LPS-stimulated induction of TNFα mRNA. Interestingly, EGCG alone slightly increased TNFα mRNA (Figs. 2A and B), which was consistent with the observed increase in TNFα protein (Fig. 1).

We examined whether the observed inhibition of LPS-mediated TNFα mRNA expression was related to a reduction in NF-κB activation. We examined NF-κB activation by detecting the active NF-κB heterodimer (p65/p50) in nuclear extracts by an EMSA. In the RAW264.7 cells, 10 mg LPS/L rapidly increased nuclear NF-κB-like DNA binding activity (Fig. 3). This activity peaked at 15 min and returned to baseline by 60 min. Pretreatment (2 h) with 100 mmol EGCG/linebhibited 10 mg LPS/L-induced nuclear NF-κB-like binding activity by about 30% (Figs. 3A and B). This finding was consistent with the observed effects of EGCG on TNFα mRNA and protein. Supershift and competition experiments were performed to confirm that the band of interest was indeed the transcriptionally active NF-κB heterodimer, p65/p50 (Fig. 4). These data clearly show that EGCG inhibits LPS-mediated NF-κB activation and subsequent TNFα gene transcription and protein synthesis.

To confirm that the above findings were not due to cytotoxic effects of EGCG, cell viability was assessed by MTT assay. At the concentrations studied, EGCG was not cytotoxic (data not shown). However, we did see a cytotoxic effect at higher concentrations (about 25% killing at 500 mmol/L). EGCG decreased TNFα production in elicited peritoneal macrophages. To test whether or not the effect of EGCG on LPS-induced TNFα production is applicable to other macrophage cells, we studied elicited murine peritoneal macrophages. Initial experiments showed that the elicited peritoneal macrophages differed from RAW264.7 cells in their sensitivity to EGCG-induced cytoxicity. At 100 mmol, EGCG killed nearly 30% of the peritoneal macrophages as assessed by MTT assay, while 50 mmol EGCG/L did not affect cell viability (data not shown). Therefore, we examined the effect of 50 mmol EGCG/L on TNFα production. The peritoneal macrophages were pretreated with 50 mmol EGCG/L for 2 h and then exposed to 10 ng LPS/Lovernight (~18 h). Similar to the RAW264.7 data, EGCG inhibited LPS-induced TNFα production by about 35% (P < 0.01 vs. control) (data not shown). Unlike the RAW264.7 cells, EGCG alone did not appreciably increase TNFα.

Green tea polyphenols inhibited LPS-induced TNFα production in vivo. We next evaluated whether the above findings were applicable to an in vivo model. The model chosen for study was the murine model of LPS-induced lethality. Rather than using EGCG, we chose to use an extracted mixture of green tea polyphenols given by oral gavage. Giving the polyphenols orally to the mice more closely simulated tea consumption by humans than did EGCG, and the gavage method allowed us to carefully control dosing. Our initial studies showed that the 1.0 g extracted green tea polyphen-
Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA as an internal control. The blots were stripped and probed for G3PDH to correct for potential differences in sample loading.

LPS /kg body wt. After 90 min, serum TNF-α levels were measured. Green tea polyphenols dramatically decreased LPS-induced serum levels of TNF-α by 55% (P < 0.05 vs. control group) and 80% (P < 0.01 vs. control group) at doses of 0.1 and 0.5 g/kg body wt, respectively (Fig. 5). No detectable TNF-α was seen in the serum of mice orally gavaged with PBS or 0.1 g or 0.5 g green tea polyphenols/kg body wt and injected with PBS. These data show that orally administered green tea polyphenols can inhibit LPS-induced TNF-α production in mice.

**Green tea polyphenols blocked LPS-induced lethality.** Finally, we examined the effect of green tea polyphenols on LPS-induced lethality in BALB/c mice. Prior studies clearly show that TNF-α is a critical factor in LPS-induced lethal shock (Beutler and Cerami 1986, Machleidt et al. 1996, Pfeffer et al. 1993; Tracey et al. 1986 and 1987). We pretreated 6–8-wk-old male BALB/c mice with either 0.1 g or 0.5 g extracted green tea polyphenols/kg body wt or an equivalent volume of PBS given by oral gavage 2 h prior to an intraperitoneal injection of either PBS containing 40 mg LPS/kg body wt or PBS alone (control). At 24 h, there were no deaths in the polyphenol-treated groups compared to 60% lethality in the control group receiving LPS alone (Table 1).

**DISCUSSION**

Our results are the first to show the protective role of green tea polyphenols against LPS-induced lethality in vivo. Our studies also support the hypothesis that green tea polyphenols block TNF-α gene expression and protein production by inhibiting NF-κB activation. These results suggest that green tea polyphenols reduce inflammatory responses by attenuating NF-κB activation.

The activation of NF-κB leads to an increase in expression of many genes whose products mediate immune responses. These include proinflammatory cytokines (e.g., IL-1, TNF-α, IL-8), enzymes (e.g., iNOS) and adhesion molecules. The production of IL-1, TNF-α, interleukin-6 (IL-6) and IL-8 are increased in acute and chronic inflammatory processes. Of these, TNF-α assumes a pivotal role. Cells of the macrophage/monocyte lineage are the predominant source of TNF-α in vivo. Though many factors stimulate TNF-α production, a major stimulus for TNF-α production in these cells is LPS. The administration of TNF-α in physiologically relevant concentrations is sufficient to mediate all of the clinical manifestations of overwhelming infection, including lethal shock and tissue injury (Tracey et al. 1986). To date, TNF-α remains the only endogenous mediator capable of eliciting all of the clinical manifestations of septic shock (Tracey et al. 1987). Blocking TNF-α activity in RAW264.7, and following pretreatment with 100 mmol EGCG/L. (B) The bands were quantified by densitometry and are presented in bar graph form for each condition.
activity using monoclonal anti-TNFα antibodies has been shown to prevent LPS-induced shock (Tracey et al. 1987), and transgenic mice deficient in the TNFα receptor are protected against lethality of exogenous LPS (Pfeffer et al. 1993). These studies show that TNFα is necessary for the development of shock and tissue injury during overwhelming bacterial infection or lethal doses of LPS. Our study clearly shows that green tea polyphenols block LPS-induced TNFα production and protect against lethal shock. Clinical studies are now focused on blocking or down-regulating TNFα in chronic inflammatory diseases. The administration of monoclonal anti-TNFα antibodies to patients with Crohn’s disease has been shown to effectively reduce disease in a significant proportion of those treated (Targan et al. 1997). Our observations warrant further investigation in the use of green tea polyphenols in the treatment of inflammatory conditions.

A number of antioxidants such as N-acetyl-L-cysteine (glutathione precursor), dithiocarbamates, vitamin E, and chelators of copper and iron ions have been reported to potently suppress NF-κB activity, suggesting that reactive oxygen species have an intracellular intermediary role (Beauparlant and Hiscott 1996, Blackwell et al. 1996). Tea has been shown to have antioxidant effects in both in vitro and in vivo systems (Salah et al. 1995). Much of the antioxidant properties of tea arise from the polyphenol fraction. In fact, 78% of the antioxidant potential of green tea extracts are accounted for by polyphenols (Salah et al. 1995). Of these, EGCG is the most abundant (40%), and a single cup (100 mL) of green tea contains approximately 50 mg of EGCG. Polyphenols potently scavenge free radicals and are also chain-breaking antioxidants. In comparison to other commonly used antioxidants, green tea polyphenols have at least twice the antioxidant potential of vitamins E or C.

Lin and Lin (1997) examined the effect of EGCG on the expression of iNOS in thioglycollate-elicited peritoneal macrophages isolated from BALB/c mice. They showed that doses of 5 and 10 mmol EGCG/L effectively inhibited iNOS expression by blocking NF-κB activation. At 10 mmol EGCG/L, LPS-mediated NF-κB activation was inhibited by nearly 40%. Those concentrations were 10% of that required to elicit a similar effect in our study. There are...
several potential differences in the two studies. These differences include the source of the EGCG extracts, how it was given and the cell models studied. Lin and Lin gave the EGCG with the LPS, whereas we pretreated the cells with EGCG for 2 h prior to LPS. Secondly, they studied thio-glycollate-elicited peritoneal macrophages, whereas we studied the murine peritoneal cell line, RAW264.7. We similarly studied murine peritoneal macrophages by intra-peritoneal injection of sterile polyacrylamide beads (Fauve et al. 1983). This method was shown to result in a more homogeneous and less activated macrophage population. These differences may explain the 10-fold difference in response. One concern is the time point Lin and Lin chose for detecting NF-κB activity. We examined the effect of EGCG on LPS-mediated NF-κB activation at a time point when nuclear NF-κB activity peaks following stimulation (15–30 min), while Lin and Lin measured NF-κB activity 3 h following stimulation. In our experiments, nuclear NF-κB activity returned to baseline by 60 min in RAW264.7 cells.

In contrast to our study, Sakagami et al. (1995) demonstrated that EGCG isolated from Japanese tea potently stimulated IL-1α, IL-1b and TNF-α synthesis in cultured human peripheral blood mononuclear cells, but not in several other human cell lines tested. They also observed that EGCG promoted adherence of these cells, though no studies were performed to determine if adhesion molecules were induced. In our study, EGCG induced a modest increase in TNF-α mRNA and protein in the RAW264.7 cells, but a similar phenomenon was not observed in either the isolated peritoneal macrophages or in serum from mice treated with green tea polyphenols. The importance/relevance of these observations and the mechanisms involved remains uncertain, but clearly appear to be cell type or cell line specific.

The concentrations used in this study are much higher than those readily available in food sources (tea). A 70 kg human would have to consume nearly 300 cups (30 L) of green tea to achieve a dose equivalent to that given to our mice in the lethality experiments. However, concentrated forms of green tea extracts are now being sold as natural supplements through many sources. Our studies demonstrate a mechanism of action that may in part explain the observed health benefits related to green tea consumption. Our findings open the door to future studies examining the pharmacological potential of green tea polyphenols in health and disease.

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**LITERATURE CITED**


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