Dietary Nucleotide Supplementation Reduces Thioacetamide-Induced Liver Fibrosis in Rats

Maria José Pérez, Antonio Suárez, José Antonio Gómez-Capilla, Fermin Sánchez-Medina and Angel Gil

Department of Biochemistry and Molecular Biology, University of Granada, Granada, Spain

ABSTRACT Dietary nucleotides reportedly promote functionality and repair in fibrotic liver. Liver fibrosis is characterized by an excessive accumulation of extracellular matrix components, which lead to the impairment of the hepatic function. The aim of this work was to evaluate the influence of dietary nucleotides on liver fibrosis induced by thioacetamide and to elucidate the mechanism by which nucleotides exert their protective effects. Rats consumed ad libitum 300 mg/L thioacetamide in drinking water and were pair-fed diets with (group TN) or without nucleotides (group TS) for 4 mo. Liver histology and extracellular matrix components, liver collagenase and prolyl 4-hydroxylase activities, and tissue inhibitor of metalloproteinases-1 were assessed. The degree of fibrosis was lower in group TN than group TS. Group TN had lower hepatic concentration of hydroxyproline (P < 0.05), collagen type I (P = 0.12) and type III (P = 0.20), fibronectin (P = 0.05), laminin (P = 0.11) and desmin (P = 0.07), higher collagenolytic activity (P < 0.05), lower prolyl 4-hydroxylase activity (P < 0.05) and lower prolyl 4-hydroxyproline (P = 0.10) and tissue inhibitor of metalloproteinase-1 (P = 0.06) expression than group TS. Moreover, expression of tissue inhibitor of the metalloproteinases-1 gene was lower in group TN than in group TS (P < 0.05). These data indicate that the reduction of liver fibrosis in nucleotide-supplemented rats may rely on the enhancement of collagenase activity and the reduction of collagen content and maturation. J. Nutr. 132: 652–657, 2002.

KEY WORDS: • liver fibrosis • dietary nucleotides • thioacetamide • rats

Regardless of its etiology, liver cirrhosis is characterized by the nodular transformation of the hepatic parenchyma and the appearance of widespread fibrosis, with an abnormal reconstruction of the lobular architecture (1). Liver fibrogenesis represents the uniform response of the liver to toxic, infectious or metabolic agents and is characterized by an excessive accumulation of extracellular matrix (ECM) components (2). Construction of hepatic sinusoids in fibrosis impairs hepatic function, increases portal vein pressure and reduces the exchange of macromolecules between the sinusoidal blood and hepatocytes (3). The end-stage of fibrogenesis is cirrhosis, which in humans, results in deteriorated organ function and life-threatening secondary sequelae (3).

The ECM composition in sepsis and cirrhotic nodules consists mainly of collagen types I and III and other noncollagenous proteins such as laminin and fibronectin (4,5). Hepatic fibrogenesis results from an imbalance between enhanced connective tissue synthesis and diminished or altered matrix breakdown (6). Both events are controlled by a complex set of proteins, including prolyl 4-hydroxylase, matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP) (5).

1 Supported by research project number ATRI-656 cofunded by Abbott Laboratories and the University of Granada in Spain.
2 To whom correspondence should be addressed. E-mail: asuarez@ugr.es.
3 Abbreviations used: ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSC, hepatic stellate cells; MMP, matrix metalloproteinase; RT-PCR, reverse transcription-polymerase chain reaction; TAA, thioacetamide; TIMP-1, tissue inhibitor of metalloproteinase-1; TNF-α, tissue necrosis factor α.

Nucleotides, the immediate precursors of nucleosides synthesized in the intestinal lumen by apical alkaline phosphatase, are an important component of diet during growth and tissue repair (7–9). Our research group recently reported the reparative effects of dietary nucleotides on experimental cirrhosis induced by thioacetamide (TAA), a model of liver cirrhosis that shares a number of metabolic and histological alterations with those found in the human disease. Dietary supplementation with nucleotides normalized protein concentration, serum amino acids as well as linoleic and arachidonic acid contents in liver microsomes (10,11). Furthermore, histological analysis of liver sections showed increased hepatocyte binuclearity and reduced extension of liver damage in rats fed nucleotides (10,12).

These preliminary observations prompted further investigation into the molecular nature of the reparative ability of dietary nucleotides in TAA-induced hepatotoxicity. We examined liver sections by optical microscopy and determined hepatic contents of hydroxyproline, total collagen, collagen type I, procollagen type III N-propeptide, fibronectin, laminin and desmin. We also studied the activity of total liver collagenases, hepatic MMP-1 (rat homologue to human MMP-1), prolyl 4-hydroxylase and TIMP-1 contents, and the expression of TIMP-1 and prolyl 4-hydroxylase α-subunit at the mRNA level.

MATERIALS AND METHODS

Materials. Female Wistar rats were supplied by the Animal Service of the University of Granada (Granada, Spain). Semipurified diets were supplied by Abbott Laboratories (Granada, Spain), and

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Dietary Nucleotides Reduce Rat Liver Fibrosis

nonpurified standard rat diet was purchased from Panlab (Barcelona, Spain). Both diets were stored at 4°C under nitrogen.

Sirus red was purchased from Gurr BDH Chemicals (Poole, England); fast green from Fluka AG (Buchs, Switzerland); neutral red, protease inhibitors, rat fibronectin and the specific antibodies rabbit anti-human fibronectin, rabbit anti-rat laminin and mouse anti-rat desmin from Sigma Chemical (St. Louis, MO). The specific antibodies rabbit anti-monkey collagen type I and rabbit anti-rat procollagen type III N-propeptide as well as rat collagen type I and rat procollagen type III N-propeptide were kindly supplied by Dr. Schuppan (Düsseldorf, Germany). Anti-N-Number rabbit anti-human immunoglobulin G antibodies conjugated to horseradish peroxidase were purchased from Bio-Rad Laboratories (Munich, Germany); rat laminin, human TIMP-1, rabbit anti-rat TIMP-1 and rabbit anti-human MMP-1 antibodies were obtained from Chemicon International, (Temecula, CA) and chicken desmin from Biodesign International (Kennebunk, ME). Moloney murine leukemia virus reverse transcriptase was purchased from Gibco BRL (Grand Island, NY); (α-32P)CTP, RNase-free DNase I, deoxyuridinetriphosphate (dNTP) and specific primers from Amersham-Pharmacia (Piscataway, NJ); AmpliTaq Polymerase from Applied Biosystems (Foster City, CA). Experimental design. All rats were housed and treated in accordance with the recommendations of the American Physiological Society (Council of Europe, 1982). Adult female Wistar rats weighing 170-190 g were housed in wire mesh cages in a temperature- and light-dark cycle. The rats were divided into three groups of 10 rats each. Two of these groups consumed ad libitum 300 mg/L TAA in drinking water for 4 mo; the other group was given water without TAA. One TAA-treated group was fed a semipurified diet (AIN-93) (13) (group T5); the other TAA-treated group was pair-fed the same semipurified diet supplemented with 3 g each of AMP, IMP, CMP, GMP and UMP per kg diet (group TF). In the semipurified diet was as follows (g/kg): casein, 220.5; cornstarch, 443.5; soybean oil, 150.0; cellulose, 80.0; L-methionine, 4.0; choline chloride, 2.0; mineral supplement, 50.0; and vitamin supplement, 0.2. The composition of the mineral and vitamin supplements was previously described (10). The group that drank water without TAA was pair-fed the nonpurified standard rat diet (group R). This group served as the reference group to obtain the analytical values of healthy rats and to verify that fibrosis was induced by TAA. The T5 and TF groups served as the control for the TN group. After 4 mo of treatment, the rats were maintained 10 additional days under the same conditions, but without TAA administration, to eliminate its acute effects. Rats were deprived of food for 12 h, anesthetized with a 250 g/L solution of urethane at a dose of 1 mL/100 g body and killed by terminal bleeding. The livers were immediately removed and a slice of the right lobule was fixed in formaldehyde solution and embedded in paraffin. The rats included all equal parts that were frozen in liquid nitrogen and stored at −80°C.

Liver histology. Paraffin blocks were cut into 5-μm thick sections and stained with periodic acid-Schiff and fast green-neutral red, and observed under a light microscope as previously described (10).

Determination of total collagen and hydroxyproline. The collagen content was measured in 15-μm thick liver sections by a colorimetric method based on the selective capacity of two dyes (siris red and fast green) to bind to collagen and noncollagenous proteins, respectively. Color equivalences were estimated in accordance with the method described previously by Jiménez et al. (14). Samples of liver were homogenized in saline solution and their proteins hydrolyzed in 6 mol/L hydrochloric acid for 16 h at 110°C. The amino acids liberated from the hydrolyzed samples were then converted to their phenylisothiocyanate derivatives which were separated and quantified by HPLC, using a Waters System (Milford, MA) as described by Fontana et al. (11).

Determination of hepatic proteins. The liver content of collagen type I, procollagen type III N-propeptide, fibronectin, laminin, desmin and TIMP-1 was quantified by an indirect sandwich ELISA as described by Somasundaram et al. (15). The content of the rat homologue to human MMP-1 was assessed using the competitive anti-human MMP-1 antibody. Total protein content in the supernatants was determined according to the method of Bradford (16). Polystyrene microtiter wells were coated overnight at 4°C with 10–250 ng of total liver protein in 50 mmol/L ammonium bicarbonate, pH 9.6. After blocking, 1:1000 dilutions of the specific antibodies and of secondary antibodies conjugated to horseradish peroxidase were added sequentially. All samples were analyzed in duplicate within the same ELISA assay. When available, solutions of purified targeted proteins were processed as the liver homogenates. With this methodology, standard curves were linear in the following ranges: from 0.5 to 50 ng for collagen type I, procollagen type III N-propeptide and TIMP-1; 6–50 ng for fibronectin and desmin; 2–10 ng for laminin. Determination of collagen concentration peak areas in each reaction tube was determined in liver homogenates with EnzCheck Collagen Assay Collagen Conjugate kit (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Collagenolytic assays used fluorescently labeled collagen type I as substrate, purified collagenase from Clostridium histolyticum as control enzyme and a general inhibitor of metalloproteinases (1,10-phenanthroline), which allowed for the quantitation of collagenase activity referred to total proteolytic activity on liver samples.

Prolyl-4-hydroxylase α-subunit and TIMP-1 mRNAs semiquantitative analysis. Semiquantitative measurement of prolyl-4-hydroxylase α-subunit and TIMP-1 gene expression was performed by a modification of the multiplex competitive polymerase chain reaction (PCR) assay (17). Total RNA was extracted from rat liver according to Chomczynski and Sacchi (18). To exclude possible contamination of cDNA with oncoviral RNA, all samples were treated with 10 U of DNase RNase-free for 30 min at 37°C. cDNA was synthesized with 1 μg of total liver DNase-treated RNA and 0.5 μg of oligo-dT18 in 20 μL of a solution containing 1X first-strand buffer, 10 mmol/L dithiothreitol, 500 μmol/L each dNTP, 1 μCi (32P)CTP, and 200 U of Moloney murine leukemia virus reverse transcriptase. After 1 h at 42°C, the efficiency of cDNA synthesis was estimated primer-extension peak areas to each reaction. Reverse-transcription (RT) reactions were performed in duplicate.

From each RT reaction, PCR reactions were carried out in duplicate with identical quantities of cDNA in all samples [estimated by (α-32P)CTP incorporation]. The competitive amplifications were made by using specific sets of primers for prolyl-4-hydroxylase α-subunit, TIMP-1 and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) within each PCR reaction. The sequence of the primers was as follows: forward primer: 5'-ATCCAGAGAGCCACTGGCCG-3' and reverse primers, 5'-TACGAGAAGGACGACTGGC-3' and 5'-ATTGAGGAGAATTCTGG-3'. For prolyl-4-hydroxylase α-subunit, GAPDH and TIMP-1 were labeled with TET (tetrazolchromo-6-carboxyfluorescein), 6-FAM (6-carboxyfluorescein), and HEX (hexachloro-6-carboxyfluorescein), respectively. Because an inverse exponential relationship between template size and efficiency has been observed (19), the lengths of the amplified products were 198, 200 and 203 bases for prolyl-4-hydroxylase α-subunit, GAPDH and TIMP-1, respectively.

When PCR reactions were performed within the exponential phase of PCR DNA synthesis, a direct correlation between initial cDNA template amount and PCR DNA production was observed (17,19). In our case, the exponential phase of the PCR reaction occurred between cycles 34 and 44 (data not shown). PCR reactions were performed in the same set with an Applied Biosystems DNA thermal cycler model 9700 as follows: an initial denaturing for 5 min at 95°C followed by 30 cycles: 94°C for 1 min, 56°C for 1 min and 72°C for 1 min, with an extension at 72°C for 5 min. The fluorescently labeled PCR products of prolyl-4-hydroxylase α-subunit, GAPDH and TIMP-1 were separated, identified by their size and distinct 5'-fluorescent label, and quantified by capillary electrophoresis in a Genetic Analyzer model 310 (Applied Biosystems). Data were expressed as the ratio of prolyl-4-hydroxylase α-subunit or TIMP-1 to GAPDH mRNAs calculated from the ratio of the fluorescent intensity in each sample to the intensity in the control sample. Statistical analysis. All results are expressed as the mean ± SEM n = 10. An unpaired Student's t test was used to compare TAA-
RESULTS

Liver histology. Chronic intoxication with orally administered TAA induced nodular transformations in liver parenchyma similar to those found in human nodular cirrhosis. New bile ducts and extended areas of fibrosis were found and fibrous septae divided the liver into pseudolobules (Fig. 1). However, regenerative nodules were not detected in group TN. Rats treated with TAA and the diet supplemented with nucleotides (group TN) had fewer histological alterations than did rats treated with TAA and fed the semipurified diet devoid of nucleotides (group TS). TAA-induced damage in the TN group was restricted to the presence of a few thin septae in the parenchyma (Fig. 1).

Extracellular matrix and severity of fibrosis. Total collagen deposits in group TN tended to be lower than in group TS (P = 0.15). Hepatic contents of ECM proteins (collagen type I, procollagen type III N-propeptide, fibronectin, laminin) and desmin, determined by ELISA, generally were higher in TAA-treated rats than in healthy rats (Table 1). The contents of all ECM proteins tended to be lower in group TN than in group TS (collagen type I, P = 0.12; procollagen type III N-propeptide, P = 0.20; fibronectin, P = 0.05; laminin, P = 0.11); and desmin, P = 0.07). When total collagen deposits, estimated as liver hydroxyproline concentration, were measured by HPLC, the results confirmed the fibrotic effect of TAA; the hydroxyproline concentration was significantly reduced in rats fed diet TN compared with those fed diet TS (P < 0.05).

ECM synthesis vs. degradation. The mechanisms by which dietary nucleotides affect extracellular connective-tissue deposition in the liver were further investigated by analyzing prolyl 4-hydroxylase, which is involved in the collagen-synthesis pathway, and liver collagensases and TIMP, which are involved in the ECM degradative route. RT-PCR analysis revealed that prolyl 4-hydroxylase α-subunit tended to be expressed at a lower level in group TN than in group TS (P = 0.10, Fig. 2A). In addition, the indirect measurement of prolyl-4-hydroxylase activity, calculated by the ratio between the hydroxyproline content and the sum of collagens type I and type III, indicated that its activity was significantly lower in group TN than in group TS (Fig. 2B).

Hepatic total collagenolytic activity was significantly higher in group TN than in group TS (Fig. 3A). The hepatic MMP-13 content tended to be higher in group TN than in

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<td>Total collagen, hydroxyproline, collagen type I, procollagen type III N-propeptide, fibronectin, laminin and desmin contents in livers of healthy (R) and orally thioacetamide-treated rats fed diets with (TN) or without (TS) nucleotides</td>
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<td>Total collagen, µg/mg protein</td>
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| Hydroxyproline, µg/g tissue      |    | 194.00 ± 18.68      | 446.24 ± 26.05      | 118.95 ± 4.33
| Collagen type I, µg/mg protein   |    | 34.67 ± 7.80        | 57.16 ± 11.52       | 325.53 ± 53.71
| Procollagen type III N-propeptide, µg/mg protein |    | 23.79 ± 1.99        | 40.69 ± 6.40        | 44.40 ± 3.24
| Fibronectin, µg/mg protein       |    | 6.83 ± 0.55         | 10.02 ± 0.92        | 36.45 ± 4.98
| Laminin, µg/mg protein           |    | 2.95 ± 0.30         | 4.18 ± 0.62         | 8.07 ± 0.89
| Desmin, µg/mg protein            |    | 25.10 ± 1.66        | 33.56 ± 2.56        | 3.38 ± 0.26

¹ Values are mean ± SEM, n = 10; ² different from TS, P < 0.05.

Rats were fed nonpurified diet and are included for reference only.
DISCUSSION

Nucleotides, the building blocks of nucleic acids, are naturally present in all foods of animal and vegetable origin as free nucleotides and nucleic acids. Nucleotide diet-derived deficiency has not been related to any particular disease. However, growing evidence indicates that dietary nucleotides may influence cell proliferation and differentiation, and modulate the physiology of the immune system, the intestine and the liver (8). Although the liver has a high capacity for nucleotide synthesis, our group has reported that adequate liver functioning may require the supply of dietary nucleotides under physiologic conditions (9). The supplementation of diets with uridine or with compounds that increase uridine synthesis in vitro and in vivo protected the liver from α-galactosamine toxicity (20). Recently, Stachlewitz et al. (21) showed that uridine may contribute to reduced α-galactosamine toxicity by preventing the activation of Kupffer cells and by diminishing tumor necrosis factor (TNF)-α production, events that are critical for toxicity. In a rat model of liver thioacetamide-induced cirrhosis, histological analysis of liver sections showed that supplementation of diets with nucleotides reduced the extension of liver damage (10). In the present study, we provide evidence that the mechanism by which dietary nucleotides protect rat liver from TAA-induced fibrosis is related to the modulation of ECM biosynthetic and degradative pathways.

Our first evidence of the beneficial effect of dietary nucleotides on liver fibrosis was the normal morphologic and macroscopic aspect of the livers in rats fed nucleotides (group TN) compared with the unsupplemented rats (group TS). Regenerative nodules were not found in group TN. Liver histological analysis confirmed that fibrotic areas (Fig. 1) and the degree of steatosis (data not shown) induced by TAA intoxication were significantly lower in rats fed nucleotides. These results indicated that the degree of liver fibrosis was lower in rats fed a nucleotide-supplemented diet.

The first noticeable molecular change in the ECM after liver injury is fibronectin accumulation in the subsinusoidal

FIGURE 2 Effect of nucleotide supplementation on hepatic prolyl 4-hydroxylase α-subunit mRNA level (A) and prolyl 4-hydroxylase activity (B) in healthy rats fed the nonpurified standard diet (group R, panel A), and in those fed a semipurified diet without (group TS, panel B) or supplemented with 3 g each of AMP, IMP, CMP, GMP and UMP per kg diet (group TN). Groups TS and TN consumed ad libitum 300 mg/L thioacetamide in drinking water. Group R was included for reference. Enzymatic activity is expressed as the ratio between liver hydroxyproline content and the sum of collagen type I and procollagen type III N-propeptide (μg/mg). Values are means ± SEM, n = 10. Different letters indicate significant differences between groups TS and TN, P < 0.05.

Group TS (P = 0.06, Fig. 3B). Liver TIMP-1 protein contents, determined by ELISA, were significantly lower in group TN than in group TS (Fig. 3C), and by RT-PCR analysis, TIMP-1 mRNA expression was lower in group TN than in group TS (Fig. 3D).

FIGURE 3 Effect of nucleotide supplementation on extracellular matrix degradation pathway in healthy rats fed the nonpurified standard diet (group R, panel A), and in those fed a semipurified diet without (group TS, panel B) or supplemented with 3 g each of AMP, IMP, CMP, GMP and UMP per kg diet (group TN). Groups TS and TN consumed ad libitum 300 mg/L thioacetamide in drinking water. Group R was included for reference. (A) Liver collagenase activity (μg/mg protein). (B) Expression of hepatic matrix metalloproteinase (MMP)-13, homologue to human MMP-1, (OD₄₅₀ units/100 mg protein). (C) Expression of hepatic tissue inhibitor of metalloproteinase-1 (TIMP-1) (mg/mg protein). (D) TIMP-1 mRNA level by reverse transcription-polymerase chain reaction analysis. Values are means ± SEM n = 10. Different letters indicate significant differences between groups TS and TN, P < 0.05.
space, followed by the synthesis, export and assembly of mature collagen fibrils and other ECM proteins, such as laminin (22). Our study is the first to examine the quantitative contribution of collagen type I and III and desmin to TAA-induced liver fibrosis deposition. As in human fibrosis (3), the analysis of liver samples showed that fibrosis was also the result of an increased deposition of collagens type I and III, desmin, fibronectin and laminin (Table 1). The hepatic content of these ECM proteins was lower in group TN (Table 1). However, these results were not as conclusive as those obtained by histological analysis, which may be due to the high variability of liver damage in tissue slices used for ELISA determinations.

Hydroxyproline is an amino acid unique to all of the collagens and represents 12% of amino acids in the major fibrillar collagen types I and III. Therefore, the measurement of hydroxyproline content by HPLC serves as an excellent standard of fibrosis (23). In our study, the hydroxyproline content was significantly decreased in rats fed nucleotides (group TN vs. group TS), which confirmed the marked reduction of collagen deposition in the hepatic ECM (Table 1).

A reduction in liver hydroxyproline content may be related to an incomplete post-translational maturation of collagen fibrils as well. The maturation of collagen fibrils involves the synthesis of hydroxylysine residues by prolyl 4-hydroxylase; these residues are needed to stabilize the collagen triple helix (24). Taking into account that type I and III collagens are the major representative members of collagenic ECM proteins in hepatic fibrosis, the ratio of hydroxyproline to collagen (sum of type I and III collagens) in liver samples may constitute indirect evidence of prolyl 4-hydroxylase activity. The calculation of its enzymatic activity showed a significant decline in group TN compared with group TS (Figure 2B). In addition to the results obtained for prolyl 4-hydroxylase α-subunit mRNA expression (Figure 2A), we hypothesized that dietary nucleotides prevent complete collagen post-translational maturation by the modulation of prolyl 4-hydroxylase synthesis and activity during liver fibrogenesis.

The mechanism of hepatic fibrogenesis is related not only to an enhanced matrix biosynthesis but also to an altered matrix breakdown (6). Chronic alcohol-induced damage to the liver in humans significantly diminishes collagenolytic activity (25), which coincides with a dramatic rise in TIMP-1 levels (26-28). In the present study, TAA intoxication also produced a net decrease in liver collagenase activity and MMP-13 (rat homologue to human MMP-1) content, and a concomitant increase in TIMP-1 expression at both protein and mRNA levels (Fig. 3).

The most interesting finding of this study was that treatment with nucleotides enhanced hepatic ECM breakdown. Total hepatic collagenase activity and MMP-13 content were significantly increased in rats fed nucleotides (Fig. 3A and 3B). In addition, the expression of TIMP-1 at both the protein and mRNA levels was lower in group TN (Fig. 3C and 3D). These data indicate that dietary nucleotides may boost liver collagenase activity through the regulation of TIMP-1 and MMP expressions.

Although the molecular mechanisms responsible for ECM accumulation in liver fibrosis are under intensive investigation (25-28), limited information is available on the precise cellular origin and on the in vivo signals that control the expression of TIMP and MMP during liver fibrosis. In culture-activated rat hepatic stellate cells (HSC), Knittel et al. (29) recently reported that the in vitro expression patterns of MMP and TIMP that resulted in either proteolytic degradation of ECM or into ECM deposition may be regulated at the transcriptional level by tissue growth factor-β1 and TNF-α. Whether the effect of dietary nucleotides on ECM accumulation is based on the transactivation of HSC or Kupffer cells and/or cytokine production will be tested in the future.

In conclusion, our data provide clear evidence that dietary nucleotides enhance degradation of ECM during TAA-induced liver fibrosis. Additional information on the potential antibiotic effect of dietary nucleotides on other animal models of liver fibrosis is required to support the potential utility of this therapeutic approach to liver fibrosis in cirrhotic humans.

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LITERATURE CITED


