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An Aqueous Stem Bark Extract of *Mangifera indica* (Vimang®) Inhibits T Cell Proliferation and TNF-induced Activation of Nuclear Transcription Factor NF-κB

Gabino Garrido[1]*, Magdalena Blanco-Molina[2], Rocío Sancho[2], Antonio Macho[2], René Delgado[1] and Eduardo Muñoz[2]

[1]Laboratorio de Farmacología, Centro de Química Farmacéutica, Apartado Postal 16042, Atabey, Playa, Ciudad de La Habana, Cuba
[2]Departamento de Biología Celular, Fisiología e Inmunología, Facultad de Medicina, Universidad de Córdoba, Avda de Menendez Pidal s/n, E-14004, Córdoba, Spain

A commercial aqueous stem bark extract of *Mangifera indica* L. (Vimang®) has been reported to have antiinflammatory, immunomodulatory and antioxidant activities. The molecular basis for these diverse properties is still unknown. This study shows that a stem bark extract of *M. indica* inhibits early and late events in T cell activation, including CD25 cell surface expression, progression to the S-phase of the cell cycle and proliferation in response to T cell receptor (TCR) stimulation. Moreover, the extract prevented TNFα-induced IκBα degradation and the binding of NF-κB to the DNA. This study may help to explain at the molecular level some of the biological activities attributed to the aqueous stem bark extract of *M. indica* (Vimang®).

Keywords: Mangifera indica; Vimang®; T cells; NF-κB.

INTRODUCTION

Recently, an aqueous stem bark extract from *Mangifera indica* L. (Anacardiaceae), which has been used in pharmaceutical formulations in Cuba under the brand name of Vimang® as an antioxidant, was reported to have potent *in vivo* and *in vitro* antiinflammatory activity (Garrido *et al*., 2001; Garrido *et al*., 2004a; 2004b), immunomodulatory effects in rat macrophages (García *et al*., 2002) and strong *in vitro* and *in vivo* antioxidant effects (Martínez *et al.*, 2000; Sanchez *et al*., 2000). Phenolic acids, phenolic esters, flavan-3-ols, mangiferin which is the predominant component of this extract (Nuñez-Sellés *et al*., 2002) and micronutrients such as selenium (Center of Pharmaceutical Chemistry, 2002) are known to be present in this extract. Various studies indicate that these compounds modulate the activities reported for this extract (Bremner and Heinrich, 2002; Singh *et al*., 2002; Guha *et al*., 1996) and inhibit mitogen-stimulated proliferation of peripheral blood T lymphocytes (Sanbongi *et al*., 1997).

The involvement of T lymphocytes activation in chronic (Liew and McInnes, 2002) and acute (Streetz *et al*., 2001) inflammation is well established (Boyton and Openshaw, 2002). The transcription factor NF-κB plays a key role in this process (for a review see Bremner and Heinrich, 2002) and the activation of the NF-κB/Rel transcription family, by nuclear translocation of cytoplasmic complexes, plays a central role in inflammation by inducing the transcription of proinflammatory genes (Makarov, 2000; Tak and Firestein, 2001).

Because the extract of *M. indica* (Vimang®) has been described to be antioxidant with antiinflammatory and immunomodulatory activities, the hypothesis was tested that these effects are mediated through its modulation of T cells proliferation and the inhibition of NF-κB. This study reports for the first time that *M. indica* extract blocks T cells proliferation and inhibits the IκBα degradation and NF-κB activation by tumor necrosis factor alpha (TNFα).

MATERIAL AND METHODS

Extract. *M. indica* was collected from a cultivated field located in the region of Pinar del Rio, Cuba. Voucher specimens (code 41722) were deposited at the Herbarium of the Academy of Sciences, guarded by the Institute of Ecology and Systematic from Ministry of Science, Technology and Environment, La Habana, Cuba. A stem bark extract of *M. indica* was prepared as a decoction and concentrated by evaporation and spray dried to obtain a fine brown powder, which was used as the standardized active ingredient of Vimang® formulations. The chemical composition of this extract has been characterized by chromatographic (planar, liquid and gas) methods, mass spectrometry and UV/VIS spectrophotometry (Nuñez-Sellés *et al*., 2002).

* Correspondence to: Dr G. Garrido, Laboratorio de Farmacología, Centro de Química Farmacéutica, Apartado Postal 16042, Atabey, Playa, Ciudad de La Habana, Cuba.
E-mail: gabino.garrido@infomed.sld.cu
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Cell lines and reagents. Jurkat cells (ATCC, Rockville, USA) were maintained in exponential growth in RPMI 1640 (Bio-Whittaker, VerViers, Belgium) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine and antibiotics (Gibco, Paisley, Scotland). The 5.1 clone (obtained from Dr N. Israël, Institut Pasteur, Paris, France) line is a Jurkat-derived clone stably transfected with a plasmid containing the luciferase gene driven by the HIV-LTR promoter and was maintained in complete RPMI medium. The HeLa–Tet-Luc is a stably transfected cell line containing both the pTet-On and pTRE2-luc plasmids, and the pSV-Hyg plasmid that confers additional resistance to hygromicin (Clontech, Palo Alto, USA). In this cell clone luciferase gene transcription is controlled by rtTA induced by doxicyclin. The stably transfected cell lines were maintained in the presence of G418 (100 μg/mL) for 5.1, and in the presence of G418 and hygromicin (100 μg/mL) for HeLa-Tet-Luc. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from ICN (Costa Mesa, USA). The anti-IκBα mAb 10B was a gift from R. T. Hay (St Andrews, Scotland), the mAb anti-tubulin was purchased from Sigma Chemical (St Louis, USA).

Isolation of human peripheral mononuclear cells (PBMC) and T cell proliferation assays. The procedure was similar to that described previously (Sancho *et al.*, 2002).

Cytofluorimetric analyses of cell surface antigen and cell cycle. PBMC cells (10^6) were stimulated with staphylococcal superantigen (SEB) (1 μg/mL) in 24-well plates in a total volume of 2 mL of complete medium for 72 h and half the cells were used for cell cycle analyses and the other half for CD25 cell surface expression measurement, according to Sancho *et al.* (2002).

Luciferase assays. To determine NF-κB dependent specific transcription, 5.1 or HeLa-Tet-Luc cells were preincubated with the *M. indica* extract followed by stimulation with TNFα (2 ng/mL) or doxicyclin (4 ng/mL) for 6 h, according to Sancho *et al.* (2002).

Western blots. This test was carried out as described by Sancho *et al.* (2002).

Isolation of nuclear extracts and mobility shift assays. This procedure was similar to that described by Muñoz *et al.* (1994).

Statistics. The results are expressed as the mean ± SD of three or four determinations, according to the experiments. Statistical analysis was performed using Student's *t*-test for unpaired data, and a value of $p \leq 0.05$ was considered to indicate a significant difference.

RESULTS

Inhibition of T cell proliferation by *M. indica* extract

Since the extract of *M. indica* (Vimang®) has been shown previously to exert some biological functions in the cells of the immune system (Sanchez *et al.*, 2000) the effect of this extract on several T cell activation events was studied. In Fig. 1A it is shown that cell treatment with the extract completely inhibited TCR-mediated cell proliferation induced by the staphylococcal superantigen SEB. Although this inhibition was found to be concentration dependent, a 90% inhibition was found with the lower doses tested (10 μg/mL). Next, the effect of *M. indica* extract on the cell surface expression of the activation markers CD25 was studied in SEB-stimulated primary T cells. In Fig. 1B it is shown that the extract at 25 μg/mL greatly inhibited the cell surface expression of this antigen. As a consequence of cell activation, primary T cells progress to the S-phase and G_2/M phases of the cell cycle, and the effect of the extract on the cell cycle progression was evaluated in SEB-stimulated T cells. SEB-treatment (1 μg/mL) induced the progression of the cell cycle with approximately 22% of the stained cells in the S or G_2/M phases, while the extract (25 μg/mL) almost completely

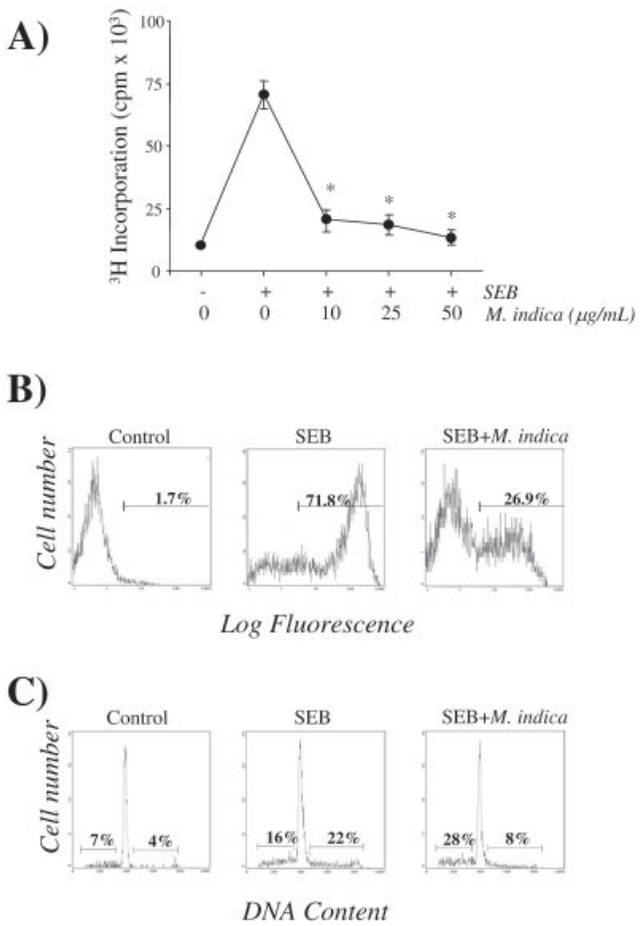

Figure 1. Effect of the extract of *M. indica* (Vimang®) in T cell activation events. (A) Purified human T lymphocytes stimulated with SEB (1 μg/mL) were pretreated with the indicated concentrations of *M. indica* extract. The results are expressed as mean ± SD cpm of triplicate measures. (B) Effect of *M. indica* extract on SEB-induced cell surface cell expression of the activation marker CD25. Human T cells were preincubated with the extract (25 μg/mL) followed by SEB (1 μg/mL) stimulation and incubated 72 h for CD25 expression. The results are representative of three different experiments. (C) Effect of *M. indica* extract on the progression of the cell cycle. Purified human T lymphocytes were stimulated with SEB (1 μg/mL) in the presence or absence of the extract (25 μg/mL) for 72 h. The percentage of cells entering the S and G_2/M phases of the cell cycle are indicated. Results are representative of three different experiments.

prevented the entry of the cells in the S-phase of the cell cycle (Fig. 1C). Interestingly, no significant differences were found in the percentage of hypodiploid cells (sub G_0/G_1) between the SEB and SEB plus the extract stimulated cells. These results indicate that at the doses used this extract was not cytotoxic or apoptotic in primary T cells.

Transcriptional activation of NF-κB is inhibited by *M. indica* extract

To explore whether or not the extract inhibited NF-κB-dependent transcriptional activity, the cloned 5.1 cell line was used that contains the luciferase gene driven by HIV-1-LTR promoter, which is responsive to TNFα through the NF-κB pathway. The cells were preincubated with increasing doses of the extract and then stimulated with TNFα for 6 h. After the indicated time, the cells were lysed, and the reporter activity was measured. As shown in Fig. 2A the extract inhibited TNFα-dependent HIV-1-LTR gene transcription. To evaluate whether the inhibitory activity of *M. indica* extract in the luciferase expression was specific or not, HeLa-Tet-Luc was preincubated with the same concentrations of the extract as that in 5.1 cells, then the cells were stimulated with doxicyclin for 6 h and the rtTA transcriptional activity measured. In Fig. 2B it is shown that the extract did not affect the luciferase expression in this control cell system.

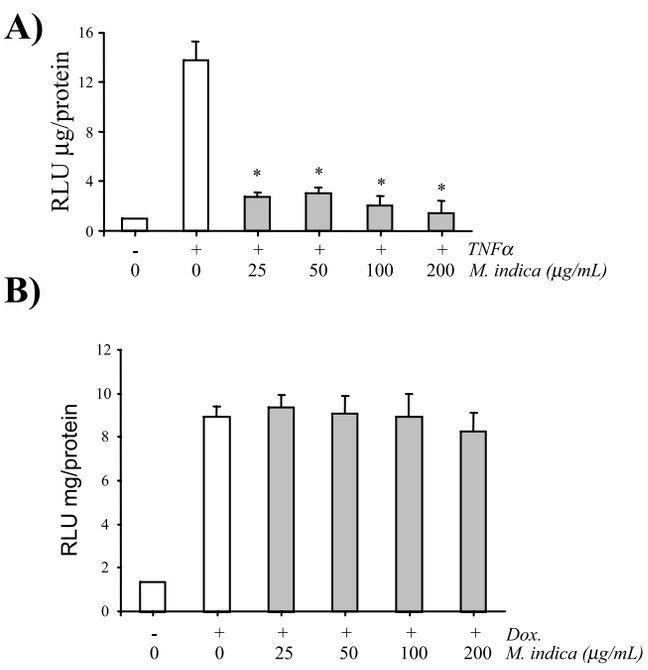

Figure 2. The extract of *M. indica* (Vimang®) is an specific inhibitor of NF-κB-dependent transcriptional activity. (A) *M. indica* extract inhibits HIV-1-LTR transactivation in 5.1 cells. The luciferase activity was measured and expressed as RLU µg/protein. The values are mean ± SD of four independent experiments. (B) *M. indica* extract does not inhibit the transactivation of the rtTA promoter induced by doxicyclin (Dox) in HeLa-Tet-Luc cells. HeLa-Tet-Luc cells were preincubated with the indicated concentrations of the extract, before stimulation with Dox (4 µg/mL). The results represent the mean ± SD of three different experiments.

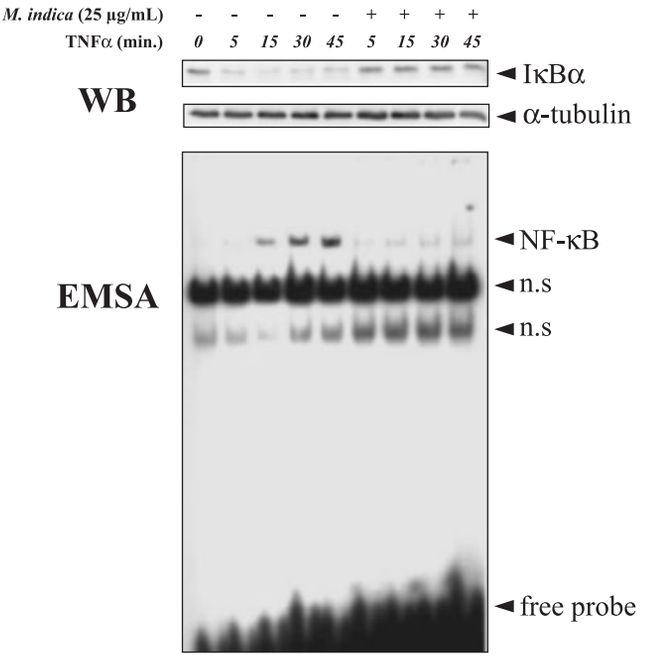

Figure 3. Effect of the extract of *M. indica* (Vimang®) on NF-κB binding to DNA and IκBα degradation. Jurkat cells were incubated with *M. indica* extract for 30 min, treated with TNFα for the indicated times and then tested for NF-κB binding (EMSA assay) and for IκBα degradation and α-tubulin expression (western blots assay, WB). Results are representative of two different experiments.

M. indica extract inhibits IκBα degradation and the binding of NF-κB to DNA

To identify the molecular target for the Vimang® effects on the NF-κB activation pathway, Jurkat cells were stimulated with TNFα (2 ng/mL) for different times in the presence or absence of the extract (25 µg/mL) and nuclear and cytoplasmic fractions analysed for NF-κB DNA binding activity by electrophoretic mobility shift assays (EMSA) and for studying the steady state level of IκBα by western blots, respectively. The kinetic experiments revealed a clear increment in the binding of NF-κB to DNA after 15 min of stimulation that was maintained throughout the time of stimulation, the increased DNA binding paralleled the degradation of IκBα that was clear after 15 min of TNFα stimulation and recovered after 60 min of stimulation. Interestingly, both the DNA binding of NF-κB and the IκBα degradation were prevented by the presence of the extract, which did not affect the steady state levels of α-tubulin (Fig. 3).

DISCUSSION

Previous studies have demonstrated that the extract of *M. indica* (Vimang®) presents a powerful scavenger activity against hydroxyl radicals and hypochlorous acid, a significant inhibitory effect on the peroxidation of rat-brain phospholipid and inhibited DNA damage by bleomycin or copper phenanthroline systems and the H_2O_2 production in peritoneal macrophages stimulated *in vivo* with TPA (Martinez *et al*., 2000; Sanchez *et al*.,

2000). Macrophage function, specifically phagocytic activity, chemotaxis in response to inflammatory stimuli and the respiratory burst were also evaluated, demonstrating that the extract has depressor effects on the phagocytic, reactive nitrogen species (RNS) and reactive oxygen species (ROS) production activities of rat macrophages (Garcia *et al.*, 2002). The extract also inhibited abdominal constriction induced by acetic acid and formalin-induced licking in mice, and the edema induced by carrageenan and formalin in mice, rats and guinea-pigs (Garrido *et al*., 2001). The inhibition of chemoattraction was also observed when the extract blocked the expression and activity of the ICAM-1 receptor in endothelial cells stimulated with IL-1 (Beltrán *et al.*, 2003). Additionally, this extract of *M. indica* reduced the production of TNFα in macrophage cell line RAW264.7 stimulated with proinflammatory stimuli interferon gamma (IFNγ) and lipopoly saccharide (LPS) (Garrido *et al*., 2004a).

Although the molecular basis for the multiple activities assigned to the extract of *M. indica* have not been defined, most of the activities inhibited by the extract require the activation of NF-κB. NF-κB is an ubiquitous transcription factor that plays a central role in the immune system by regulating many inflammatory responses through transcriptional activation of certain pro-inflammatory cytokines, the inducible nitric oxide synthase (iNOS) gene and other genes involved in inflammation (Verma *et al*., 2004).

Phenolics (like those present in the extract of *M. indica*) have provided numerous examples of compounds with antiinflammatory activity mediated by inhibition of NF-κB in various cell types (Surh *et al*., 2001). Black tea (*Thea sinensis*) derivatives, theaflavin and catechin polyphenols, were studied for their ability to suppress NF-κB activation in LPS-stimulated RAW267.4 cells. Other compounds, such as epigallocatechin-3-gallate, have been shown to possess NF-κB inhibitory activity against TNFα-induced activation in normal human epidermal keratinocytes and human epidermal carcinoma (A431) cells (Bremner and Heinrich, 2002; Ahmad *et al*., 2000).

M. indica extract also contains between 0.03% and 0.08% of selenium (Center of Pharmaceutical Chemistry, 2002). Selenium (Se) is an essential component of the enzyme glutathione peroxidase and thioredoxin reductase families, important antioxidant enzymes that catalyse the reduction of hydroperoxides produced from oxidized species such as superoxide and lipoperoxides (Holben and Smith, 1999). Moreover, Se modulates the activity of NF-κB in LPS-treated human T-cells and lung adenocarcinoma cells via a different mechanism involving the redox state of specific cysteine residues of NF-κB and selenoproteins, such as thioredoxin (Kim and Stadtman, 1997). Furthermore, the cytoprotective role of thioredoxin in modulating the DNA binding of transcription factors such as NF-κB, AP-1, p53 and PEBP-2 (polyomavirus enhancer-binding protein-2) coincides with the increased translocation of this protein into the nucleus upon subjecting the cells to inflammatory stress (Harper *et al*., 2001). Our results suggest that the extract could also modulate the NF-κB activation by the Se content in the extract. As the concentrations of selenium in patients with rheumatoid arthritis are relatively low (Darlington and Stone, 2001), and the importance of the inhibition of the NF-κB activation in this disease has been demonstrated (Makarov, 2000; Tak and Firestein, 2001), the extract could be useful as a supplementary source of Se in these patients.

M. indica extract could be of therapeutic interest to develop drugs that are able to interfere with the overexpression of the cytokines and the activity of NF-κB.

This study provides evidence for a possible mechanism by which the aqueous stem bark extract of *M. indica*, known in Cuba under the brand name of Vimang®, has been used as an antiinflammatory and immunomodulatory agent by inhibiting the NF-κB transcription factor and the T cell proliferation induced by superantigen.

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