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RAPID COMMUNICATION

INHIBITION OF TUMOR NECROSIS FACTOR BY CURCUMIN, A PHYTOCHEMICAL

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Abstract --- Curcumin, contained in the rhizome of the plant *Curcuma longa* Linn, is a naturally occurring phytochemical that has been used widely in India and Indonesia for the treatment of inflammation. The pleiotropic cytokine tumor necrosis factor-α (TNF) induces the production of interleukin-1β (IL–1), and, together, they play significant roles in many acute and chronic inflammatory diseases. They have been implicated in the pathogenesis of intracellular parasitic infections, atherosclerosis, AIDS and autoimmune disorders. This report shows that, *in vitro*, curcumin, at 5 μM, inhibited lipopolysaccharide (LPS)-induced production of TNF and IL-1 by a human monocytic macrophage cell line, Mono Mac 6. In addition, it demonstrates that curcumin, at the corresponding concentration, inhibited LPS-induced activation of nuclear factor kappa B and reduced the biological activity of TNF in L929 fibroblast lytic assay.

Key words: inflammation; tumor necrosis factor; NFκB; interleukin-1; antioxidant; macrophages

Curcumin is a polyphenol that is contained in the rhizome of the plant *Curcuma longa* Linn. In the form of the herbal powder turmeric, it has been used for centuries as an anti-inflammatory remedy in Asian medicine. With the recent renewed interest in the pharmaceutical potential of natural products, between 1992 and 1994, over 40 studies that explored the biomedical potential of curcumin have been published. For example, curcumin reduces the release of reactive oxygen species by stimulated neutrophils and inhibits the activation of AP-1, a TPA+-inducible transcription factor that regulates TNF production and protein kinase C activity [1-5].

At the initiation of inflammation, NFκB is one of the transcription factors that are activated. It up-regulates TNF production, and TNF induces the production of IL-1. The pleiotropic pro-inflammatory cytokines TNF and IL-1 affect nearly every tissue and organ system and induce the expression of a variety of genes and proteins that induce acute and chronic inflammation. Whereas NFκB up-regulates TNF production,

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+Abbreviations: BHA, butylated hydroxyanisole; IL-1, interleukin-1β; LPS, lipopolysaccharide; NAC, *N*-acetyl-cysteine; NFκB, nuclear factor kappa B; TNF, tumor necrosis factor-α; and TPA, *O*-tetradecanoylphorbol-13-acetate.

TNF activates NFκB reciprocally. IL-1 and TNF also potentiate each other's production [6]. Hence, these factors form an autocrine loop that escalates their own levels and, consequently, the pathogenesis of many inflammatory diseases.

Recently, Kopp and Ghosh [7] reported that the anti-inflammatory compounds sodium salicylate and aspirin, which are known for inhibiting prostaglandin synthesis, also inhibit NFκB activation at high concentrations, 10-20 mM. Similarly, the anti-inflammatory effect of curcumin has been associated with its inhibition of cyclooxygenase, lipoxygenase and prostaglandin synthesis [8]. The present report describes the effect of curcumin on the early events in the course of inflammation. It shows that curcumin inhibited NFκB activation, reduced the production of TNF and IL-1 by human macrophages *in vitro*, and suppressed the lytic biological activity of TNF.

MATERIALS AND METHODS

Experimental design. The human monocytic macrophage cell line Mono Mac 6 [9] was cultured in complete RPMI-1640 (Gibco/Life Technologies, Grand Island, NY), which contained 100 units/mL of penicillin, 100 μg/mL of streptomycin, 2 mM glutamine, 0.01 mM sodium pyruvate, 0.1 mM Minimum Essential Medium non-essential amino acids, 0.05 mM 2-mercaptoethanol and 10% fetal bovine serum (Hyclone, Logan, UT). Curcumin (Kalsec, Kalamazoo, MI) was prepared as a 100 mM stock solution in acetone or ethanol and then was diluted to 5 and 10 μM in the culture medium to which 0.5% solvent had been added. It was then added to cells at equal volume so that the final concentration of antioxidants was as desired and solvent was at 0.25%. LPS (4 μg/mL, Sigma, St. Louis, MO) was added 30-60 min after the addition of curcumin.

TNF and IL-1 assays. After 4 hr of incubation, the supernatants were collected and the concentrations of TNF were determined by TNF ELISA (Biosource International, Camarillo, CA). For IL-1, the supernatants were collected after 18 hr, and the concentrations of IL-1 were determined by the IL-1 ELISA (Endogen, Boston, MA).

NFκB gel mobility analysis. Nuclear extracts were prepared as described in Andrews and Faller [10], except that 0.6% Nonidet P-40 was added to the cell lysis buffer. NFκB activity was determined using the gel shift assay system (Promega, Madison, WI). The sequence of the NFκB oligonucleotide used was 5'-AGT TGA GGG GAC TTT CCC AGG C-3'.

Viability assay. Viability tests were performed as described in Mosmann [11]. The experiment was performed as described above except that LPS, which may induce apoptosis, was not added. At 4 and 18 hr of incubation, the cells were washed three times to remove the antioxidants before dimethylthiazol-diphenyltetrazolium bromide was added.

TNF cytolytic assay. The assay was performed as described in Ref. 12 with the L929 cell line, which is one of the prototypic cell lines for measuring the cytotoxic activity of TNF.

RESULTS AND DISCUSSION

LPS is one of the most extensively studied inducers of TNF and IL-1 production. In this series of experiments, it was used to induce TNF and IL-1 production and NFκB activation in Mono Mac 6 cells. For monocytes and macrophages, TNF production was apparent at 4 hr and subsided by 18 hr after the addition of LPS; therefore, the level of TNF production was determined at 4 hr after activation. At 5 μM, curcumin reduced the production of TNF by about 57%, from 528 ± 88 to 227 ± 74 pg/mL (Fig. 1).

NFκB regulates the expression of many genes that are related to inflammatory responses. As an inactive form, it is bound to an inhibitor that is named inhibitor of NFκB (IκB) and resides in the cytoplasm. In response to LPS or TNF activation, it is released and translocated from the cytoplasm into the nucleus. In this report, the

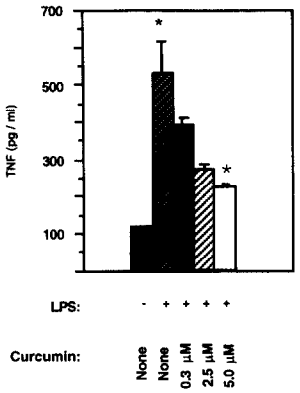

Fig. 1. Effect of curcumin on TNF production. Human Mono Mac 6 cells (2 x 10^5 /mL) were cultured in the presence of the indicated concentrations of curcumin and LPS (4 µg/mL). After 4 hr, supernatants were collected and the concentrations of TNF were determined by ELISA. Values are means ± SD, N=3. To compare the difference between the positive controls and the samples with 5.0 µM curcumin, statistical analysis was performed by Student's *t*-test, and the level of significance (*) was determined at $P < 0.05$.

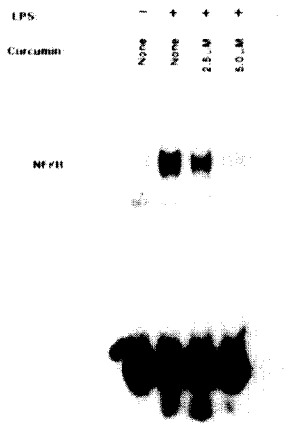

Fig. 2. Effect of curcumin on nuclear translocation of NFκB transcription factor. Nuclear extract was prepared from a total of 10^7 Mono Mac 6 cells that were cultured at 10^5 cells/mL with LPS and curcumin as described in Materials and Methods. After 4 hr, the nuclei were extracted, and a gel mobility assay was performed.

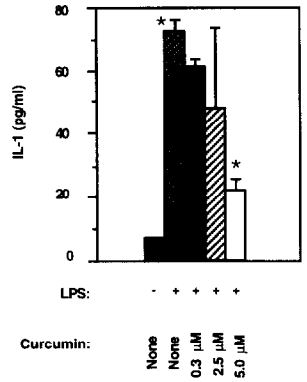

Fig. 3. Effect of curcumin on IL-1 production. The experiment was performed as described in Fig. 1, except that supernatants were collected after 18 hr, and the concentrations of IL-1 were determined by ELISA.Values are means ± SD, N=3. To compare the difference between the positive controls and the samples with 5.0 µM curcumin, statistical analysis was performed by Student's *t*-test, and the level of significance (*) was determined at $P < 0.05$.

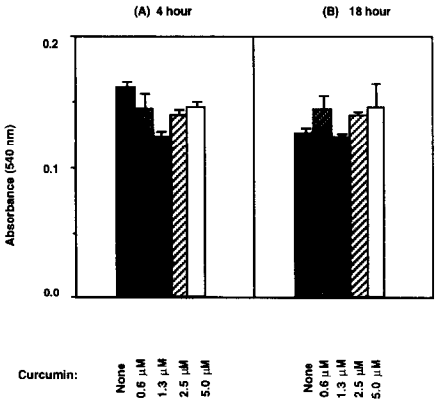

Fig. 4. Effect of curcumin on cellular toxicity. Cells were cultured in the presence of curcumin, which was diluted to the desired concentrations in culture medium to which 0.25% alcohol had been added. After 4 and 18 hr, viability was determined as described in Materials and Methods. Values are means ± SD, N=3. Statistical analysis was performed as described in the legend of Fig. 1.

effect of curcumin on the activation of NFκB was analyzed by detecting the translocation of NFκB into the nucleus, as indicated in a gel mobility assay on nuclear extract. Ziegler-Heitbrock *et al.* [9] have shown that the level of NFκB in nuclear fractions of Mono Mac 6 cells is similar from 1 to 8 hr after addition of LPS. In this report, nuclei were extracted at 4 hr after LPS addition, corresponding to the time when TNF production was measured. Curcumin, at 2.5 and 5 μM, inhibited activation of NFκB (Fig. 2). At 5 μM, curcumin reduced LPS-stimulated nuclear translocation of NFκB to the level of the unstimulated Mono Mac 6 human macrophages. The specificity of NFκB binding was verified by competitive inhibition with the NFκB oligonucleotide sequence and by non-competitive inhibition with OCT1 oligonucleotide sequence (5'-TGT CGA ATG CAA ATC ACT AGA A-3') (data not shown). This result suggested that one of the mechanisms by which TNF production is reduced is through the inhibition of gene transcription. Further mechanistic studies will decipher whether other processes may also be involved.

Unlike TNF, 70% of IL-1 is released from the cells in 24 hr; therefore, IL-1 production was determined at 18 hr [13]. Corresponding to the TNF study, curcumin reduced IL-1 production. About 69% reduction was achieved at 5 μM, from 72.8 ± 3.4 to 22.3 ± 3.0 pg/mL (Fig. 3). The decrease in TNF production may have contributed to this decrease in IL-1 production.

Two viability assays verified that the reduction of both TNF and IL-1 was not due to general cellular toxicity of curcumin. The trypan blue exclusion assay showed that the cells remained intact (data not shown). Tetrazolium salt assays showed that the cells were metabolically active after incubation with curcumin for 4 hr or 18 hr (Fig. 4). As controls, antioxidants were also added to empty wells that did not contain cells to verify that after washing, there was no residual activity of the antioxidant to hydrolyze the tetrazolium salt.

Inflammation results in the production of oxidative free radicals, which are activators of NFκB and TNF production. One of the most well-defined characteristics of curcumin is its antioxidative property. Structurally, it has a diketone group and two phenol rings that act as electron traps to prevent hydrogen peroxide production and to scavenge hydroxyl and superoxide radicals. In addition, two or more molecules of curcumin can conjugate to chelate iron [3].

Several reports have shown that some other antioxidants, besides curcumin, may also down-regulate NFκB and TNF. NAC, a precursor of glutathione, which reduces hydroxy radicals and peroxides, is a well-established clinical medicine that is known to inhibit NFκB activation [14]. When tested in parallel with curcumin, we found that NAC reduced TNF production by about 72% at 60 mM, from 45.5 ± 0.2 to 12.8 ± 0.7 pg/mL. There was more than a thousand-fold difference between the efficacy of curcumin (5 μM) and that of NAC (60 mM). Other antioxidants, such as BHA, desferrioxamine, *o*-phenanthroline and dithiocarbamate, inhibit NFκB activation at 100-300 μM [15,16]. The most effective NFκB inhibiting agent reported in the literature before this report was membrane-bound pentamethyl-6-hydroxychromane, a derivative of vitamin E. Similar to curcumin, it reduces activation at 10 μM in Jurkat T cells [17]. Vitamin E and curcumin, as antioxidants, are more potent than BHA in reducing lipid peroxidation of sheep erythrocyte membranes; moreover, they also inhibit TNF signal transduction [3,17]. Possibly, the ability of curcumin to scavenge free radicals may not be the only mechanism of its action.

The effect of curcumin on the lytic function of TNF was investigated. TNF may mediate a variety of biological activities. Among them, TNF lysis of actinomycin-D-treated L929 fibroblasts has been one of the standard bioassays for quantitating TNF, although the signal transduction pathway of TNF seems to be cell line-dependent [6,12]. At 25 to 50 μM, curcumin inhibited the cytotoxicity of L929 fibroblasts when 4 hr LPS-stimulated Mono Mac 6 supernatant was added as a source of TNF (Fig. 5A). Figure 5B shows that, at 50 μM, curcumin was capable of neutralizing the lytic activity of 100-500 ng/mL of recombinant TNF by 30-40%. TNF transduces signals through several possible pathways that may involve G proteins, phospholipase A_2, arachidonate, lysophospholipase, protein kinase C and free radicals as effector molecules [6]. It has been observed that TNF-mediated killing is oxygen dependent, free radical facilitated, and blocked by inhibitors of cyclooxygenase [6]. Curcumin has high efficacy probably because it may block these pathways, possibly at multiple sites. On the other hand, having to block multiple pathways can also explain why higher

concentrations of curcumin are needed for the inhibition of TNF-mediated lysis than for the reduction of TNF production.

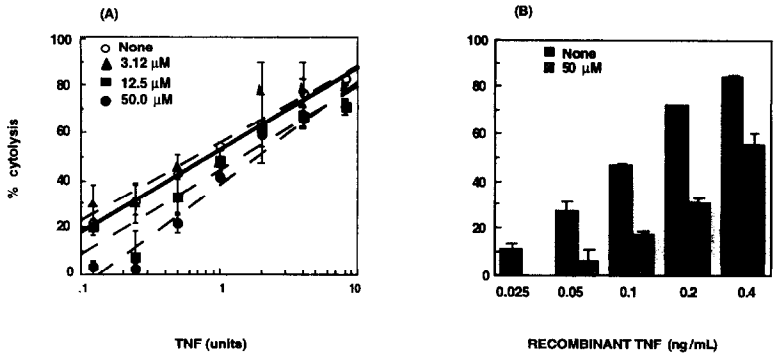

Fig. 5. Effect of curcumin on the biological activity of TNF. (A) TNF contained in supernatant from LPS-stimulated Mono Mac 6 cells or (B) recombinant TNF was added to L929 fibroblasts, and curcumin was added at 3.1, 12.5 and 50 μM. For the LPS supernatant, the amount of supernatant that produces 50% lysis of fibroblasts was standardized as containing 1 unit of TNF activity. TNF activity was indicated by lysis of the fibroblasts. Percent lysis = 100 - (absorbance of experimental well / absorbance of well in which TNF was omitted). Values are means ± SD, N=3.

This report widens the scientific base for this time-tested Asian anti-inflammatory agent, curcumin. The efficacy reported here is similar to the concentrations (5-15 μM), detected by others for *in vitro* inhibition of protein kinase C activity, AP-1 transcription factor activation, and EBV-DR promoter, SV40 promoter enhancer, and HIV-LTR mediated transcription [2,4,5,18]. AIDS patients have elevated levels of TNF in their sera, and HIV has binding sites for NFκB on its long terminal repeats (LTR) [19,20]. Thus, the TNF inhibitors pentoxifylline and thalidomide are being used in clinical trials as a means for slowing the progression, although not effecting the cure, of AIDS [21,22]. Curcumin has also been used in a preliminary trial in HIV-seropositive individuals and found to increase CD-4 and CD-8 cell counts [23]. TNF, IL-1 and NFκB play roles in the pathogenesis of many disorders, e.g. AIDS, septic shock syndrome, arthritis, tuberculosis, malaria, and atherosclerosis [4,19]. The information reported here suggests that the possibility of developing curcuminoids for some of these inflammatory diseases is worth exploring. Many reports have shown that curcumin is effective on skin, and in the circulatory and gastrointestinal systems [8,24-27]. Yashi *et al.* [24] have shown that it decreases serum triglycerides and phospholipids. Huang *et al.* [8,25] have shown that when applied topically curcumin can reduce TPA-induced skin thickening and taken orally can decrease the incident of colon hyperplasia. However, the pharmacokinetics of curcumin remains incompletely understood, and there are indications that its biological effects may be mediated by a metabolite, tetrahydrocurcumin [3]. Our data also showed that, *in vitro*, the effective concentration of curcumin is high and has a narrow window; cellular toxicity was detected for Mono Mac 6 cells at 20 μM, although L929 fibroblasts remained unaffected at 50 μM (data not shown). Therefore one may also contemplate the possibility that a derivative is preferred for drug development.

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REFERENCES

1. Limasset B, le Doucen C and Dore JC, Effects of flavonoids on the release of reactive oxygen species by stimulated human neutrophils. Multivariate analysis of structure-activity relationships (SAR). *Biochem Pharmacol* **46**: 1257-1271, 1993.
2. Bouvier G, Hergenhahn M, Polack A, Bornkamm GW and Bartsch H, Validation of two test systems for detecting tumor promoting EBV inducers: Comparative responses of several agents in DR-CAT Raji cells and in human granulocytes. *Carcinogenesis* **14**: 1573-1578, 1993.
3. Ho C-T, Osawa T, Huang M-T and Rosen RT, *Food Phytochemicals for Cancer Prevention II: Tea, Spices and Herbs.* American Chemical Society, Washington, DC, 1994.
4. Huang T-S, Lee S-C and Lin J-K, Suppression of c-Jun/Ap-1 activation by an inhibitor of tumor promotion in mouse fibroblast cells. *Proc Natl Acad Sci USA* **88**: 5292-5296, 1991.
5. Liu J-Y, Lin S-J and Lin J-K, Inhibitory effects of curcumin on protein kinase C activity induced by 12-O-tetradecanoyl-phorbol-13-acetate in NIH 3T3 cells. *Carcinogenesis* **14**: 857-861, 1993.
6. Aggarwal BB and Vileck J, *Tumor Necrosis Factors: Structure, Function, and Mechanism of Action.* Marcel Dekker, New York, 1992.
7. Kopp E and Ghosh S, Inhibition of NF-κB by sodium salicylate and aspirin. *Science* **265**: 956-959, 1994.
8. Huang M-T, Lysz T, Ferraro T, Abidi TF, Laskin JD and Conney AH, Inhibitory effect of curcumin on *in vitro* lipoxygenase and cyclooxygenase activities in mouse epidermis. *Cancer Res* **51**: 813-819, 1991.
9. Ziegler-Heitbrock HWL, Sternsdorf T, Liese J, Belohradsky B, Weber C, Wedel A, Schreck R, Bauerle P and Strobel M, Pyrrolidine dithiocarbamate inhibits NFκB mobilization and TNF production in human monocytes. *J Immunol* **151**: 6986-6993, 1993.
10. Andrews NC and Faller DV, A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res* **19**: 2499, 1991.
11. Mosmann T, Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* **65:** 55-63, 1983.
12. Hogen MM and Vogel SN, Measurement of tumor necrosis factor α and β. In *Current Protocols in Immunology* (Eds. Coligan JE, Kruisbeak AM, Margulies DH, Shevach EM and Strober W), pp. 6.10.1-6.10.5. Wiley, New York, 1991.
13. Lonnemann G, Endres S, van der Meer JW, Cannon JG, Koch KM and Dinarello CA, Differences in the synthesis and kinetics of release of interleukin-1 alpha, interleukin-1 beta and tumor necrosis factor from human mononuclear cells. *Eur J Immunol* **19**: 1531-1536, 1989.
14. Staal FJT, Roederer M, Raju PA, Anderson MT, Ela SW, Herenberg LA and Herzenberg LA, Antioxidants inhibit stimulation of HIV transcription. *AIDS Res Hum Retroviruses* **9**: 299-306, 1993.
15. Israel N, Gougerot-Pocidalo M-A, Aillet F and Virelizier J-L, Redox status of cells influences constitutive or induced NFκB translocation and HIV long terminal repeat activity in human T and monocytic cell lines. *J Immunol* **149**: 3386-3393, 1992.
16. Schreck R, Meier B, Mannel DN, Droge W and Baeuerle PA, Dithiocarbamates as potent inhibitors of nuclear factor κB activation in intact cells. *J Exp Med* **175**: 1181-1194, 1992.
17. Suzuki YJ and Packer L, Inhibition of NF-kappa B activation by vitamin E derivatives. *Biochem Biophys Res Commun* **193**: 277-283, 1992.
18. Li CJ, Zhang LJ, Dezube BJ, Crumpacker CS and Pardee AB, Three inhibitors of type 1 human immunodeficiency virus long terminal repeat-directed gene expression and virus replication. *Proc Natl Acad Sci USA* **90**: 1839-1842, 1993.
19. Lahdevirta J, Maury CP, Teppo AM and Repo H, Elevated levels of circulating cachectin/tumor necrosis factor in patients with acquired immunodeficiency syndrome. *Am J Med* **85**: 289-291, 1988.
20. Duh EJ, Maury WJ, Folks TM, Fauci AS and Rabson AB, Tumor necrosis factor α activates human immunodeficiency virus type 1 through induction of nuclear factor binding to the NFκB sites in the long terminal repeat. *Proc Natl Acad Sci USA* **86**: 5974-5978, 1989.
21. Biswas DK, Dezube BJ, Ahlers CM and Pardee AB, Pentoxifylline inhibits HIV-1 LTR-driven gene expression by blocking NFκB action. *J Acquir Immune Defic Syndr* **6**: 778-786, 1993.
22. Makonkawkeyoon S, Limson-Pobre RNR, Moreira AL, Schauf V and Kaplan G, Thalidomide inhibits the replication of human immunodeficiency virus type 1. *Proc Natl Acad Sci USA* **90**: 5974-5978, 1993.
23. Copeland R, Baker D and Wilson H, Curcumin therapy in HIV-infected patients initially increased CD-4 and CD-8 cell counts. *Int Conf AIDS* **10**: 216, 1994.
24. Yashi S, Imaizumi K, Nakamura M, Aimoto J and Sugano M, Effects of *Curcuma xanthorrhiza* Roxb. and curcuminoids on the level of serum and liver lipids, serum apolipoprotein A-1 and lipogenic enzymes in rats. *Food Chem Toxicol* **31**: 213-218, 1993.
25. Huang M-T, Deschner EE, Newmark HL, Wang Z-Y, Ferraro TA and Conney AH, Effect of dietary curcumin and ascorbyl palmitate on azoxymethanol-induced colonic epithelial cell proliferation and focal areas of dyplasia. *Cancer Lett* **64**: 117-121, 1992.
26. Ravindranath V and Chandrasekhara N, Metabolism of curcumin--Studies with [^{3}H]curcumin. *Toxicology* **22**: 337-344, 1982.
27. Holder GM, Plummer JL and Ryan AJ, The metabolism and excretion of curcumin (1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) in the rat. *Xenobiotica* **8**: 761-768, 1978.