

Effect of Aloe vera on nitric oxide production by macrophages during inflammation.(Research Paper)



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OBJECTIVE : To demonstrate the mechanism of action mediating the acute and chronic antiinflammatory activity of leafy exudate of Aloe vera (AVL) in animal models of inflammation. **MATERIALS AND METHODS :** The acute antiinflammatory activity of AVL was evaluated using carrageenan and dextran as phlogistic agents while its chronic antiinflammatory effect was investigated in a complete Freund's adjuvant-induced model of arthritis. The degree of inflammation in all models was measured plethysmographically. The effect of AVL on nitric oxide production in mouse peritoneal macrophages was measured by the Griess reagent. **RESULTS :** AVL (25 mg/kg) significantly reduced carrageenan and dextran-induced pedal edema in rats by 61.9% and 61.7%, respectively. In the Freund's adjuvant-induced model of chronic inflammation, AVL showed chronic antiinflammatory activity but failed to decrease the arthritic index indicating the absence of antiarthritic activity. AVL (10 [micro]g/ml) caused a decrease in NO production in macrophages without causing toxicity.

CONCLUSION : AVL possesses acute and chronic antiinflammatory activity, which is partly mediated by reduced production of NO, which in turn prevents the release of inflammatory mediators.

Introduction

Inflammation is generally considered as an essentially protective response to tissue injury caused by noxious physical, chemical or microbiological stimulus. It is a complex process involving various mediators, such as prostaglandins, leukotrienes and platelet activating factor.[1] The major macrophage derived inflammatory mediators such as pro-inflammatory cytokines, tumour necrosis factor- α (TNF- α) and the reactive free radical nitric oxide (NO) synthesized by inducible NO synthase (iNOS), contribute to the development of inflammatory diseases.[2] Thus, inhibition of the excessive production of TNF- α and/or NO could be employed as criteria to evaluate potential antiinflammatory compounds. The current management of inflammatory diseases is limited to the use of antiinflammatory drugs whose chronic administration is associated with several adverse effects. Plant-derived products are slowly emerging as a viable alternative because they are cheap, abundantly available and relatively less toxic.

The genus Aloe belongs to the Asphodelaceae family, Alooideae subfamily, and comprises about 420 species of succulent plants. They are indigenous to southern and

eastern Africa and Madagascar but have been introduced in other tropical countries, the Mediterranean area and the West Indies.[3] Aloe has been used as a folk medicine for 3000 years and in the last decade Aloe vera is being extensively used in health drinks, topical creams, toiletries and cosmetics.[4] From the leaves of Aloe plants, three types of commercial products are obtained namely, the dried exudate, excreted from the aloin cells present in the zone of the vascular bundle, the gel, a mucilaginous juice present in the centre of the leaf and the oil, extracted by organic solvents.[3] The dried exudate is used as a bitter in alcoholic beverages while the oil, comprising the fatty fraction of the leaf is used primarily in the cosmetic industry as a pigment carrier and soothing agent. Studies have shown that the gel component has antiinflammatory activity[5] that is mediated through antibradykinin[6] activity and inhibition of prostaglandin production.[7] However, no studies have been undertaken with regard to the antiinflammatory activity of the leaf exudate. Accordingly, in this study, we have evaluated the antiinflammatory potential of Aloe vera leaf exudate as also demonstrated that this antiinflammatory activity is mediated partly via reduction of nitric oxide production in macrophages.

Materials and Methods

Animals

Male albino Wistar rats (100-120 g) and Swiss albino mice (av. wt. 25-30 g) were used. All animals were housed under standard conditions of temperature (25[+ or -]5[degrees]C). A 12-h light/dark cycle was maintained and the animals were provided with standard pellet diet and water ad libitum. They were acclimatized for one week and then subdivided for different experimental schedules. The experiments were carried out after getting approval from the Institutional Animal Ethics Committee.

Chemicals and drugs

Naphthyl ethylene diamine dihydrochloride [NED (Loba Chemie)], sulphanilamide, zymosan, phenazine methosulphate [PMS (SRL)], MTS: {3-(4, 5 dimethylthiazol-2-yl)-5-(carboxymethoxyphenyl)-2-(4- sulphophenyl)-2H tetrazolium (Promega), Indomethacin (Inmecin 25, E.M. Pharmaceuticals Pvt. Ltd., India), dexamethasone (Dexona, Cadila Healthcare Ltd., India), Complete Freund's Adjuvant, Carrageenan and dextran were obtained from Sigma, MO, USA.

Preparation of plant extract

Fresh succulent leaves of Aloe vera were collected, the inner gel component removed and the leafy exudate homogenized in an electric grinder. This was subsequently lyophilized and stored at 4[degrees]C. It was solubilized in dimethylsulfoxide (DMSO, 50 mg/ml), labelled as AVL and used for all experiments.

Tests for acute inflammation

Carrageenan or dextran-induced edema

Animals were divided into four groups (n = 6). In all the groups, acute inflammation was

induced by injection of either 0.1 ml of freshly prepared carrageenan (1.0 %) in 0.9 % w/v NaCl solution[8] or 0.1 ml of dextran (2%) in 0.9% w/v NaCl solution[9] into the subplantar region of the hind paw of rats. AVL in different doses (0- 25 mg/kg, b.w.) and indomethacin (2.5, 5 and 10 mg/kg, b.w., as a standard reference) were orally administered one hour before injection of the phlogistic agent. The paw volumes were measured plethysmographically (using water) once prior to administration of the phlogistic agent and thereafter at hourly intervals for 3 h. The percentage of inhibition was calculated as follows[10]

$E_c - E_t$

$\times 100$

E_c

where E_c is the edema volume of the control group and E_t is the edema volume of the treated group.

Test of chronic inflammation

Freund's complete adjuvant induced arthritic model

Rats were divided into three groups (n = 6). Experimental arthritis was induced according to the method of Newbould et al .[11] Briefly, 0.1 ml of Freund's complete adjuvant was injected intradermally into the plantar aspect of the hind paw of each animal. Animals were administered AVL (25 mg/kg, b.w., orally) and dexamethasone (0.1 mg/kg, b.w., orally, as a standard reference) for the initial 13 days. The degree of inflammation was measured plethysmographically; accordingly, edema formation and the percentage of inhibition was calculated as described above on days 1, 3, 5, 9, 13 and 21 and the primary and the secondary lesions were measured.

Primary lesions refer to the edema formation in the injected hind paw that peaks 3-5 days after injection of the phlogistic agent and is measured on day 5 by calculating the percent inhibition of the edema volume of the injected paw using the formula described above. Secondary lesions are immunologically mediated changes characterized by inflammation of the non-injected sites (hindleg, forepaw, ears nose and tail) decrease in weight and occur after a delay of 11-12 days. Accordingly, secondary lesions were evaluated by calculating the percent inhibition of paw volume of the non-injected right paw over control on day 21 and using an arthritic index as the sum of scores according to the method of Schorlemmer.[12]

Lesion site Nature of lesion Score

Ear a. Absence of nodules and redness. 0

b. Presence of nodules and redness. 1

Nose a. No swelling of connective tissue. 0

b. Intense swelling of connective tissue. 1

Tail a. Absence of nodules. 0

b. Presence of nodules. 1

Forepaw a. Absence of inflammation. 0

b. Inflammation of at least one joint. 1

Hind paw a. Absence of inflammation. 0

b. Slight inflammation. 1

c. Moderate inflammation. 2

d. Marked inflammation. 3

Preparation of mouse peritoneal macrophages To elicit activated peritoneal macrophages, mice were injected i.p. with starch [2% in phosphate buffered saline (PBS), 0.02 M, pH 7.2, 2 ml/mice]. After three days, the peritoneal cells comprising principally macrophages were lavaged with sterile PBS (10 ml). The exudate was centrifuged at 4000 rpm for 10 min and the resultant cell pellet washed in PBS and finally resuspended in complete RPMI-1640 phenol red free medium supplemented with 10% fetal calf serum (FCS).[13] Macrophage viability (>95%) was confirmed by the Trypan blue dye exclusion technique.

Measurement of nitric oxide (NO) production in macrophages

Macrophages obtained by peritoneal lavage as described above were seeded (5×10^6 sub/ml) in RPMI 1640 phenol red free medium supplemented with 10% FCS in Petri dishes (35 mm diameter) and incubated at 37[degrees]C, 5% CO₂ for 4 hrs. AVL (0 - 10.0 [micro]g/ml) was added and incubated for an additional 48 hrs. At the end of 48 h, 0.5 ml of the supernatant was withdrawn and nitrite accumulation as an indicator of NO production was measured using Griess reagent.[14] Briefly, 0.5 ml of Griess reagent [mixture of 1:1 of naphthylethylenediamine dihydrochloride (0.1% in water) and sulphanilamide (1% in 5% phosphoric acid)] was added to 0.5 ml of supernatant and incubated in the dark at room temperature (25-30[degrees]C) for 10 min. Finally, absorbance at 546 nm was measured spectrophotometrically and a standard curve using sodium nitrite was used to calculate concentrations of nitrite. To demonstrate specificity, macrophages were incubated in the presence of N-monomethyl arginine [(L-NMMA), 0.1mM] an established inhibitor of NO production.

Cytotoxicity assay

To determine the viability of macrophages in the presence of AVL or DMSO, they were seeded in 96-well tissue culture plates (1×10^6 sub cells/ml of RPMI 1640, supplemented with 10% FCS, 0.2 ml per well) and incubated with either AVL (0 - 10 mg/ml) or DMSO (0-

1%) alone for 48 hrs. At the end of drug exposure, the MTS assay was performed.[15] Briefly, MTS [$\{3-(4, 5 \text{ dimethylthiazol-2-yl})-5-(\text{carboxymethoxy-phenyl})-2-(4\text{-sulphophenyl})-2\text{H tetrazolium}\}$] was prepared (2 mg/ml in PBS) and stored in dark at -20 [degrees]C. Another stock solution of phenazine methosulphate (PMS) was prepared (0.92 mg/ml in PBS) and stored -20[degrees]C. Just before use, MTS and PMS were mixed in the ratio of 20:1 and 20 [micro]l of the solution was added to each well. The plates were then incubated at 37[degrees]C for 3 hrs and absorbances measured at 490 nm using a Multiskan ELISA reader (BioRad, USA).

Statistical analysis

Data were expressed as mean[+ or -]SEM and statistically assessed using one-way ANOVA followed by Tukey test; $P < 0.05$ was considered significant.

Results

AVL possesses strong acute antiinflammatory activity in carrageenan and dextran-induced animal models of inflammation

AVL at 25 mg/kg, b.w., showed significant antiinflammatory activity in both carrageenan and dextran models. In the control group, paw volume was maximum at the third hour after which the paw volume decreased gradually and therefore readings up to the third hour were recorded [Table 1][Table 2]. However, the antiinflammatory activity of AVL was sustained up to the sixth hour (data not shown). Indomethacin served as a positive control and was initially tested at three different concentrations of 2.5, 5.0 and 10.0 mg/kg, b.w. With 2.5 and 5.0 mg/kg, b.w., a maximum of 19.53% and 21.65% reduction in paw edema, respectively, was obtained. However, with 10 mg/kg, b.w., a marked reduction of 75% was obtained and accordingly, this concentration was selected for all experiments.

Chronic antiinflammatory activity of AVL in a complete Freund's adjuvant-induced model of arthritis.

A significant decrease in the primary lesion as evident on day 5 was observed with both AVL (33.3%, $P < 0.05$) and the dexamethasone treated group (57.69%, $P < 0.007$) [Table 3] [Figure 1] as compared to controls. With regard to the secondary lesions, it could only be partially evaluated as no edema formation was evident in the contralateral hind paw of control animals. On the 21st day, a significant decrease in edema volume was observed in the injected paw of both AVL (52.7%, $P < 0.01$) and dexamethasone-treated group (63.51%, $P < 0.005$).

With regard to the mean arthritic index, the score of AVL-treated animals was not different from that of the control group being 2.0 vs. 1.8, respectively, indicating that AVL does not possess antiarthritic activity. As expected, the dexamethasone-treated group showed profound antiarthritic activity since it decreased the arthritic index almost 10-fold being 0.17.

AVL-treated macrophages showed decreased nitric oxide (NO) production

The formation of NO is classically assayed by measuring nitrite (NO₂⁻) production, a primary, stable and nonvolatile breakdown product of NO. The Griess reagent uses sulphanilamide and NED under acidic conditions to yield an azo compound that can be measured spectrophotometrically. AVL caused a 43.64% decrease in NO production in murine macrophages as compared to control values being 31.0 [micro]M vs. 55.0 [micro]M. The marked decrease in NO production in the presence of L-NMMA (8.75 [micro]M) confirmed specificity of the reaction.

Toxicity of AVL in macrophages

As AVL decreased NO production in macrophages, it was relevant to confirm that this decrease was not due to AVL or DMSO induced cell death but specifically by macrophage inactivation. Accordingly, macrophages were incubated with AVL or DMSO and cell viability was determined by the MTS assay. Since this conversion can only be accomplished by viable cells, the amount of formazan is a direct measure of the number of cells present.[14] It was found that DMSO up to 0.5% was nontoxic to macrophages [Figure 2]A, $p=0.72$. However, with 1.0% DMSO, a significant increase in toxicity was observed [Figure 2]A, $P<0.0001$. AVL up to a concentration of 0.1 mg/ml showed no decrease in viability [Figure 2]B, $P=0.31$ but with higher concentrations (>0.25 mg/ml), a significant decrease in viability was observed [Figure 2]B, $P<0.0001$. Accordingly, all experiments were performed using a maximum of 0.1% DMSO or 0.1 mg/ml AVL.

Discussion

The inhibition of carrageenan-induced inflammation in rats is an established model to screen compounds for potential antiinflammatory activity. According to Vinegar et al . (1987),[16] the development of carrageenan-induced edema is biphasic; the first phase occurs within one hour of carrageenan administration and is attributed to the release of cytoplasmic enzymes, histamine and serotonin, from the mast cells. The second phase (>1.0 h) is mediated by an increased release of prostaglandins in the inflammatory area and continuity between the two phases is provided by kinins. With regard to dextran-induced edema, it has been reported to be mediated mainly by histamine and 5-HT released by the mast cells.[17] Taken together, it suggests that as AVL possesses potent acute antiinflammatory activity [Table 1][Table 2], it is mediated possibly due to inhibition of the synthesis and/or release of mediators of inflammation, principally the prostaglandins. This can only be defined after developing models of inflammation using specific mediators.

The immunologically mediated complete Freund's adjuvant arthritic model of chronic inflammation is considered as the best available experimental model of rheumatoid arthritis.[18] This method in rats was originally developed by Pearson et al . (1963),[19] wherein an injection of complete Freund's adjuvant in the rat hind paw induces inflammation. It has been proposed that bacterial peptido-glycan and muramyl dipeptide are important nonspecific immunogenic contributory components. In this model of immunologically mediated chronic synovial inflammation and arthritis, macrophages play a central role. After activation they are capable of synthesizing mediators such as PGE₂ and cytokines such as TNF- α and IL-1. In turn, these synthetic products induce the production

of a variety of enzymes which initiate cartilage and bone destruction.[20], [21]

NO is an important physiological messenger and effector molecule in many biological systems, including immunological, neuronal and cardiovascular tissues.[22] Nitric oxide, in macrophages, is produced as a free radical by iNOS by catalyzing the oxidation of guanidino nitrogen of L-arginine, thereby converting L-arginine to L-citrulline.[22] NO is an important signalling and effector molecule in inflammation and immunity as it is known to couple with superoxides to form peroxynitrite. These, in turn, induce the production of prostaglandin endoperoxide synthase from monocytes/macrophages resulting in enhanced synthesis of prostaglandins, established mediators of inflammation.[23] The reduced NO production by macrophages in the presence of AVL suggests that AVL by decreasing the release of NO, inhibits the release of inflammatory inhibitors such as prostaglandins resulting in suppression of inflammation. This can be confirmed by measuring the expression of iNOS in macrophages; such studies are ongoing.

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References

1. Gryglewski RJ. Molecular mechanisms of inflammation. *Eur J Rheumatol Inflamm* 1981;4: 153-9.
2. Freeman BD, Natanson C. Antiinflammatory therapies in sepsis and septic shock. *Expert Opin Investigl Drugs* 2000;9:1651-63.
3. Saccu D, Bogoni P, Procida G. Aloe Exudate: Characterization by reverse phase HPLC and Headspace GC-MS. *J Agric Food Chem* 2001;49:4526-30.
4. Atherton P. Aloe vera : Magic or medicine? *Nurs Stand* 1998;41:49-54.
5. Reynolds T, Dweck AC. Aloe vera leaf gel: A review update. *J Ethnopharmacol* 1999;68:3-37.
6. Bautista-Perez R, Segura-Cobos D, Vasquez-Cruz B. In vitro antibradykinin activity of Aloe barbadensis gel. *J Ethnopharmacol* 2004;93:89-92.
7. Langmead L, Makins RJ, Rampton DS. Anti-inflammatory effects of Aloe vera gel in human colorectal mucosa in vitro . *Aliment Pharmacol Ther* 2004;19:521-7.
8. Winter CA, Risley EA, Nuss GW. Carrageenin induced edema in hind paw of the rats as an assay for anti inflammatory drugs. *Proc Soc Exp Biol Med* 1962;111:544-7.
9. Rowley DA, Benditt EP. 5-hydroxytryptamine as mediators of vascular injury provided

by agents which damage mast cells in rats. *J Exp Med* 1956;130:399-415.

10. Tsai CC, Lin CC. Anti-inflammatory effects of Taiwan folk medicine 'Teng-Khia-U' on carrageenan- and adjuvant-induced paw edema in rats. *J Ethnopharmacol* 1999;64:85-9.

11. Newbould BB. Chemotherapy of arthritis induced in rats by mycobacterial adjuvant. *Br J Pharmacol* 1963;21:127-36.

12. Schorlemmer HU, Kurrle R, Schleyerbach R, Bartlett RR. Disease modifying activity of malononitrilamides, derivatives of leflunomide's active metabolite, on models of rheumatoid arthritis. *Inflammation Research* 1999;48:113-4.

13. Hudson L, Hay FC. *Practical Immunology*. 3rd ed. Oxford, London: Blackwell Scientific Publications; 1989.

14. Hibbs JB Jr., Taintor RR, Vavrin Z, Rachlin EM. Nitric oxide: A cytotoxic activated macrophage effector molecule. *Biochem Biophys Res Commun* 1988;157:87-94.

15. Barltrop JA, Owen TC, Cory AH, Cory JG. 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl) tetrazolium, inner salt (MTS) and related analogs of 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) reducing to purple water soluble formazans as cell-viability indicators. *Bioorg Med Chem Lett* 1991;1:611-4.

16. Vinegar R, Truax JF, Selph JH, Johnston PR, Venable AL, McKenzie KK. Pathway to carrageenan-induced inflammation in the hind limb of the rat. *Fed Proc* 1987;46:118-26.

17. Lo TN, Almeida AP, Beaven MA. Dextran and carrageenan evoke different inflammatory responses in rat with respect to composition of infiltrates and effect of indomethacin. *J Pharmacol Exp Ther* 1982;221:261-7.

18. Williams RO. Rodent models of arthritis: relevance for human disease. *Clin Exp Immunol* 1998;114:330-2.

19. Pearson CM, Wood FD. Studies of arthritis and other lesions induced in rats by the injection of mycobacterial adjuvant. VII. Pathological details of the arthritis and spondylitis. *Am J Pathol* 1963;42:73-95.

20. Hopkins SJ. Cytokines and eicosanoids in rheumatic diseases. *Ann Rheum Dis* 1990;49:207-10.

21. Jadot G, Michelson AM, Puget K. Anti-inflammatory activity of superoxide dismutases; Studies on adjuvant induced polyarthritis in rats. *Free Radic Res Commun* 1986;2:27-42.

22. MacMicking J, Xie Q, Nathan C. Nitric oxide and macrophage function. *Annu Rev Immunol* 1997;15:323-50.

23. Landino LM, Crews BC, Timmons MD, Morrow JD, Marnett LJ. Peroxynitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis. *Proc*

Natl Acad Sci USA 1996;93:15069-74.