Basic Studies

Allicin, the active component of garlic, prevents immune-mediated, concanavalin A-induced hepatic injury in mice


Abstract: Background/Aim: Allicin, the immunologically active component of garlic, has been found to affect oxidative stress and immune response in several experimental systems. In the present study, we examined the ability of allicin to prevent immune-mediated, concanavalin A (Con A)-induced liver damage in mice. Methods: Mice were pretreated with allicin for 7 days before their inoculation with Con A (15 mg/kg). The serum levels of liver enzymes and liver histology were examined 24 h after Con A administration. The effect of Con A and allicin on serum levels of tumor necrosis factor-α (TNF-α) and nuclear factor-κB (NF-κB) activation in the liver were examined 2 h after Con A administration, in a separate group of rats, and the effect of allicin on Con A-induced expression of inducible nitric oxide synthase (iNOS) was determined by western blot analysis 24 h after Con A injection. Results: The histopathologic damage in the mouse livers, and the Con A-induced increase of aminotransferases and TNF-α were markedly inhibited in the mice pretreated with allicin before Con A injection (P<0.01). NF-κB binding activity to the nucleus, which increased 2 h after Con A administration, was attenuated by allicin. The expression of iNOS protein which was induced following Con A administration was significantly attenuated by allicin. In vitro studies showed that allicin inhibited TNF-α-mediated T cell adhesion to extracellular matrix components and to endothelial cells. Allicin also inhibited TNF-α-mediated intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression on human vascular endothelial cells. Conclusions: This study demonstrates that immune-mediated liver damage in mice can be prevented by allicin, probably because of its immunomodulatory effects on T cells and adhesion molecules and inhibition of NF-κB activation.

Garlic (Allium sativum) has been considered a beneficial therapeutic agent for many years (1). Several studies in humans have demonstrated that garlic has beneficial effects on blood pressure (2, 3), platelet aggregation (4), and fibrinolytic activity (5). Likewise, favourable effects of garlic on serum lipid levels (6, 7) and on atherosclerosis (8) have also been investigated. Allicin (2-propene-1-sulfinothioic acid S-2-propenyl ester) is the main biologically active component of freshly crushed garlic extracts. Its biological activity was attributed to either antioxidant activity or thiol disulfide exchange. The antioxidant properties of both allicin and its precursor, alliin (+S-allyl-L-cysteine sulfoxide) were investigated using the spin trapping technique. It was found that both compounds possessed significant antioxidant activity and that the biological effect of allicin is in part because of its rapid reaction with thiol-containing proteins (9). The ability of allicin to scavenge hydroxyl radicals was demonstrated using high performance liquid chromatography (HPLC) (10). It was also reported that aged garlic extract creates antioxidant properties by highly water-soluble organosulfur compounds, such as S-allylcysteine and S-allylmercaptocysteine, and inhibits the activation of nuclear factor-κB (NF-κB), protects against free radical-mediated...
damage to DNA and against radiation-induced damage (11).

It has also been recognized that garlic compounds can affect certain aspects of the inflammatory response. Several studies have indicated that garlic-related compounds modulate the activities of a number of inflammatory cells including T cells and macrophages. For example, in human T cells, garlic-related compounds inhibited NF-κB activation induced by both tumor necrosis factor-α (TNF-α) and H₂O₂ (12), as well as the expression of inducible nitric oxide synthase (iNOS) in activated macrophages (13). Furthermore, in Balb/c mice infected with leishmaniasis, garlic exhibited immunomodulatory properties elucidated by shifting the cytokine response to a T helper 1-type pattern thereby causing an enhanced protective effect (14). It has been shown that garlic extracts have a potent inhibitory effect on neutrophil migration through endothelial cell monolayers (15). In the present study, we wished to further explore the immunomodulatory effects of allicin, the active component of garlic, in vitro and in an in vivo model of immune cell-mediated, acute hepatitis induced in mice by the lectin concanavalin A (Con A). Con A is a T cell mitogen that activates T lymphocytes in vitro and induces T cell-dependent liver damage in mice. The induction of hepatitis by Con A depends on the interaction between CD4⁺ T helper cells and macrophages (16), and on the production of proinflammatory cytokines, in particular, TNF-α (17, 18). Importantly, Con A-induced hepatic damage can be prevented by polyclonal anti-TNF-α antibodies (18) and by recombinant soluble TNF receptors (19).

In this study, we demonstrated that in Con A-treated mice the increase of serum aminotransferases, TNF-α, and hepatic necroinflammation, were markedly attenuated by allicin. The mode of action may be explained by the inhibition of NF-κB activation and of iNOS expression observed in the in vivo experiments and by our in vitro studies, which demonstrated inhibition of T cells adhesion to the extracellular matrix and endothelial cells, and attenuation of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression in endothelial cells in response to TNF-α.

Materials and methods

Induction and evaluation of liver damage

Allicin was produced as a pure product by the reaction of immobilized garlic allinase with a preparation of synthetic alliin ((+)-S-2-propenyl L-cysteine S-oxide), identical in its chemical properties with the natural alliin, according to Mirelman et al. (20). The purity and concentration of the allicin obtained was determined by HPLC as previously reported (9, 21). For the experiments described in this study, the concentration of allicin used was 1.8–2.0 mg/ml in phosphate buffer 50 mmol/l pH 6.5. The pure allicin solution, kept at 4 °C, was relatively stable during the week of the experiment and the concentration was routinely determined.

Balb/c male mice (weight 25 g) were maintained at the Animal Breeding Center of the E. Wolfson Medical Center. Treatment of the animals was in accordance with institutional guidelines. Acute liver injury was induced by injecting 6–8-week-old mice with Con A 15 mg/kg (Sigma, St. Louis, MO) in 250 μl of phosphate-buffered saline (PBS) via the tail vein. Twenty-four hours after the administration of Con A, the mice were bled, euthanized with chloral hydrate anesthesia, their abdomens opened by a midline incision and sections from the left liver lobe were excised for histopathologic examination. Liver sections were fixed in a 5% neutral formol solution and stained with hematoxylin and eosin. Histopathology changes including centrilobular necrosis and lobular inflammatory infiltration were graded on a severity scale from 0 to 3 (0 = no lesion, 1 = mild, 2 = moderate, 3 = severe) (22).

Allicin (300 μl/mouse/day) was administered orally by gavage for 7 days before Con A administration. The last dose of allicin was administered 1 h prior to Con A injection. A dose of 300 μl/mouse/day equals to 0.54 mg/mouse (21 mg/kg/day). Allicin doses at the same range (8–20 mg/kg/day) were as effective as enalapril in decreasing blood pressure, insulin and triglycerides levels in fructose fed rats (23).

Biochemical assessment of liver injury

The extent of the liver damage was estimated, in addition to a histopathological examination, by determining the serum levels of alanine aminotransferase (ALT) with an automated Monarch Monoanalyzer 2000 (Allied, Lexington, MA).

Determination of serum levels of TNF-α

For determination of TNF-α levels, blood was drawn from mice 2 h after administering Con A. Serum TNF-α concentrations were assayed by enzyme-linked immunosorbent assay kits (Genzyme-Corp., Cambridge, MA) for quantitation of mouse TNF-α, as described by the manufacturer. Each sample was tested in duplicate.
T cell adhesion studies

Materials
Anti-human ICAM-1 and VCAM-1 monoclonal antibodies were purchased from Serotec (Oxford, UK). Recombinant human ICAM-1 and VCAM-1 (R&D systems, Minneapolis, MN); purified human laminin (Sigma Chemicals, St. Louis, MO); purified human fibronectin (Chemicon, Temecula, CA); PMA (Sigma Chemicals): Na$_5$Cr$_{20}$O$_{4}$ (Dupont-NEN; Boston, MA). Reagents for cell culture were purchased from Beit Haemek (Israel) unless stated otherwise.

Isolation of T cells
T lymphocytes were isolated as previously described (24). Briefly, the mononuclear cells were isolated on Ficoll gradients, then washed and incubated for 2 h, 37 °C, 7.0% CO$_2$ in a humidified incubator. Nonadherent cells were collected and incubated on nylon wool columns (Novamed, Ltd., Jerusalem, Israel). Unbound cells were eluted by extensive washing with PBS, and the resulting cell population was >92% T lymphocytes.

Cell adhesion assays
T cell adhesion to endothelial cell ligands, extracellular matrix (ECM) or ECM ligands was determined as described (24). Flat-bottomed wells in microtiter dishes were either precoated with fibronectin (10 µg/ml), laminin (10 µg/ml), ICAM-1 (10 µg/ml) or VCAM-1 (10 µg/ml) for 1 h at 37 °C. After extensive washings, the wells were blocked with 0.1% bovine serum albumin (BSA). $^{51}$Cr-labeled T cells ($10^5$ cells/100 – 1 RPMI 1640 containing 0.1% BSA) were added to the wells, and the plates were incubated at 37 °C for 1 h. Nonadherent cells were removed by repeated washing with PBS, and adherent cells were lysed with 1 M NaOH/0.1% Triton X-100, then radioactivity was measured in a $\gamma$-counter (Packard, Ramsey, MN).

Enzyme-linked immunosorbent assays (ELISA)
ICAM-1 and VCAM-1 on cell surfaces were quantified by ELISA as described (25). Human vascular endothelial cells were cultured to confluency and pretreated with allicin (0–100 µM) for 24 h at 37 °C. Pretreated cells were then incubated with fresh growth medium, which contained TNF-α (50 U/ml) for 12 h. After this incubation, the cells were washed with PBS and fixed with 1% paraformaldehyde for 15 min, washed with 2% BSA at 4 °C, and then were incubated with monoclonal antibodies against either ICAM-1 or VCAM-1 for 2 h at 37 °C. The concentration of each antibody was 1 µg/ml. Next, the cells were washed three times in PBS with 0.05% Tween 20 (PBS-T) and incubated for 2 h at 37 °C with a second biotinylated antibody, and a streptavidin-biotinylated horseradish peroxidase complex was added (Amersham, Sydney, Australia). Orthophenylene-diamine served as substrate for the horseradish peroxidase complex. We measured optical density with an ELISA reader. Test and control samples were assessed in triplicate in each experiment, a total of three experiments were performed.

Determination of NF-κB activation
Isolation of nuclear proteins
Nuclear proteins from liver tissue were isolated by the modified method of Dignam (26). Briefly, frozen tissues were weighed, transferred to Correx tubes, and homogenized with an Ultra-Turrax homogenizer in ice-cold hypotonic buffer (1.5 mM MgCl$_2$, 10 mM KCl, 0.2 mM PMSF, 1.0 mM dithiothreitol (DTT), 10 mM HEPES, pH 7.9). Homogenates were incubated for 10 min on ice and centrifuged (25 000 g, 15 min, 4 °C; RC5C Sorvall Instruments, Du Pont, Newtown, CT). Cytoplasmic proteins were collected from the supernatant and nuclear proteins from the pellets. Pellets were washed once and centrifuged at 10 000 g for 15 min at 4 °C, after which the pellets were suspended in ice-cold low salt buffer (25% (v/v) glycerol, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 0.2 mM PMSF, 1.0 mM DTT, 1.2 M KCl, 20 mM HEPES, pH 7.9) drop by drop to make a final concentration of 0.4 M KCl. Samples were incubated on ice for 30 min, with smooth shaking. Soluble nuclear proteins were recovered by centrifugation (25 000 g, 30 min, 4 °C) and proteins were stored at −80 °C. Finally, the concentration of total protein in the samples was measured with the Protein Assay Reagent kit of Bio-Rad.

Electrophoresis mobility shift assay
A consensus DNA-binding oligonucleotide of NF-κB target genes ($5^\prime$-AGT TGA GGG GAC TTT CCC AGG-3$'$) was used as a probe and labeled using a random priming kit (Promega Corp., Madison, WI) in the presence of (α-$\gamma$-32P)-deoxyctydine triphosphate (3000 µCi/mmol, Amersham Pharmacia Biotech, Buckinghamshire, UK). Binding reactions, containing 35 pmol (1.75 pmole/µl) of oligonucleotide and 1 µg of nuclear protein were conducted at room temperature for 20 min in a total volume of 10 µl in binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM MgCl$_2$, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol (v/v), and 0.5 µg poly dI:dC).
For competition reactions, unlabeled oligonucleotide was added 5 min before adding a radiolabeled probe. After the binding reactions, gel-loading buffer was added and the reaction was subjected to nondenaturating 4% polyacrylamide gel electrophoresis (PAGE) in 0.25 × TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) at 100 V/20 mA for 2 h. Next, the gels were vacuum dried and exposed to X-ray film (Kodak, Biomax MS, New Haven, CT) at −70 °C. The specificity of the binding was ascertained by competition with a 100-fold molar excess of unlabeled consensus oligonucleotides.

**Determination of iNOS**

iNOS protein expression was studied by western blot in four groups of mice (normal, untreated mice, allicin only, Con A only and Con A + allicin) 24 h after Con A injection (15 mg/kg). Allicin (300 μl = 21 mg/kg/mouse/day) was administered orally by gavage for 7 days before Con A administration. iNOS was detected by western blot (27, 28) in supernatants of crude liver homogenates. Livers were homogenized using Ultrathurrax homogenizer in PBS solution. Samples containing 40 μg protein were separated on 7.5% SDS PAGE gels. Proteins were electrophoretically transferred to nitrocellulose. Nonspecific sites were blocked with a solution containing 5% BSA for 1 h at room temperature. Then the membrane was incubated with mAB antimouse NOSII antibody (Biotest, Kfar Saba, Israel) at a 1:750 dilution in TBS containing 5% BSA for 1 h at room temperature, with antimouse IgG peroxidase (1:1000 dilution). The blots were washed and protein visualized using the enhanced chemiluminescence method (Amersham, Sydney, Australia) according to the manufacturer’s instructions. Mouse anti actin monoclonal antibody was purchased from Chemicon (Temecula, CA). The density of the corresponding bands was quantitated with a BIO-RAD imaging densitometer (Ramsey, MN).

**Statistical analysis**

The data are presented as means ± SD. The significance of differences between different experimental groups was determined by ANOVA, followed by posthoc pairwise testing using Bonferroni’s method.

**Results**

Inhibition of the Con A-induced ALT increase by allicin

Administration of allicin at a dose of 300 μl/mouse for 7 days prior to Con A administration was effective in preventing Con A-induced hepatitis, as assessed by the serum levels of ALT (Fig. 1A, \(P < 0.001\)).

Inhibition of the Con A-induced TNF-α increase by allicin

Pretreatment with allicin inhibited the Con A-induced increase in the serum levels of TNF-α, as measured 2 h after Con A administration. (Fig. 1B, \(P < 0.01\)).
Inhibition of the Con A-induced hepatic necroinflammation by allicin

Pretreatment with allicin inhibited the Con A-induced pericentral inflammation and centrilobular necrosis observed 24 h after Con A administration (Fig. 1C, \( P \leq 0.001 \)).

Inhibition of T cell adhesion to ECM ligands and to adhesion molecules by allicin

As shown in Figs 2 and 3, allicin inhibited TNF-mediated ICAM-1 and VCAM-1 expression on human vascular endothelial cells (Fig. 2) and T cell adhesion to ECM and to ECM components (fibronectin and laminin), and to endothelial cells (Fig. 3). To show that allicin can also inhibit adhesion molecules in murine cells we examined the effect of allicin on murine T cell adhesion directly to the ligands ICAM-1 and VCAM-1. As shown in Fig. 4, allicin inhibited the PMA-stimulated adhesion of murine T cells to ICAM-1 and VCAM-1 in a dose-dependent manner (\( P < 0.01 \)).

Effect of allicin on liver histology in Con A hepatitis

As shown in previous studies, liver sections of control mice fixed 24 h after Con A administration and examined microscopically, exhibited inflammatory infiltration around the central veins and large areas of centrilobular necrosis (Fig. 5A). In contrast, in mice treated with allicin before Con A administration, the liver damage was minimal: no areas of intralobular necrosis or significant inflammatory infiltration were observed (Fig. 5B). Magnification \( \times 160 \).

Inhibition of Con A-induced NF-κB activation by allicin

To study the effect of Con A administration on NF-κB binding activity, we performed electrophoresis mobility shift assays. Although no specific NF-κB binding activity was detected in the unstimulated state (data not shown), Con A injection induced NF-κB binding activity as determined after 2 h. This binding activity was attenuated in the livers of the allicin-pretreated mice (Fig. 6).

Inhibition of Con A-induced iNOS expression by allicin

The effect of allicin on iNOS expression was determined by western blot analysis in liver homogenates from the following groups: no treatment, allicin treatment, Con A only, or Con A + allicin. As shown in Fig. 7, the Con A-induced expression of iNOS was significantly reduced by allicin.
In this study, we demonstrated that allicin has a protective effect on immune-mediated, Con A-induced hepatitis in mice. In allicin-treated mice that were inoculated with Con A, the elevation in serum levels of liver enzymes and TNF-α were abolished, NF-κB activation and iNOS expression were inhibited and the hepatic necroinflammation was much improved. In vitro studies demonstrated the inhibitory effect of allicin on T cell adhesion to the ECM and to endothelial cells and on the expression of ICAM-1 and VCAM-1 on endothelial cells, in response to TNF-α.

Allicin and garlic-related compounds, can affect certain aspects of the inflammatory response, probably independent of their known antioxidant activity (14, 15). Several studies have indicated that garlic-related compounds modulate the activities of a number of inflammatory cells including T cells and macrophages. In the present study, which was the first to elucidate the anti-inflammatory effects of allicin in the liver in an in vivo model of immune-mediated hepatitis, we demonstrated that allicin attenuated Con A-induced liver damage, iNOS expression and NF-κB activation. Previous studies have shown that NF-κB activation is a main regulatory transcription factor in the expression of iNOS in Con A hepatitis, and that inhibitors of NF-κB such as pyrrolidine dithiocarbamate or silibinin decreased NF-κB translocation and attenuated liver damage in this model (29, 30). This is consistent with the demonstrated inhibition of Con A-induced iNOS expression by allicin, observed in our in vivo study.

It has been shown previously in human T cells that garlic-related compounds inhibit NF-κB activation induced by both TNF-α and H₂O₂.
These observations suggest that garlic-related molecules might negatively affect the severity and duration of inflammatory reactions in vivo.

The mode of action of allicin in the prevention of Con A-induced liver damage may involve down-regulation of the production of TNF-α by T lymphocytes and macrophages, since TNF-α is a central mediator of hepatic inflammation in Con A-induced hepatitis (18, 19). An alternative possibility is that the anti-inflammatory effects of allicin are because of its known antioxidant and free radical scavenging activity, which was demonstrated in other experimental models (9, 10), and may indirectly affect the production and secretion of TNF-α. Another possible mechanism for the hepatoprotective effects of allicin-related compounds in Con A-induced hepatitis is the inhibition of the migration of CD4⁺ T lymphocytes from blood vessels and penetration of the subendothelial ECM. Since induction of experimental immune-mediated hepatic damage requires extravasation and migration of activated T cells, allicin could prevent the early events in the evolution of Con A-induced liver damage.

In our studies, we demonstrated that allicin suppressed the expression of key endothelial adhesion molecules after human vascular endothelial cells were stimulated with the proinflammatory cytokine TNF-α. The expression of these adhesion molecules is a hallmark event in the development of the proinflammatory state in endothelial cells. This proinflammatory state is an early step in the inflammatory cascade that promotes the attraction, adherence, and subendothelial migration of mononuclear cells to the liver.

There are at least two mechanisms by which free radicals are involved in the regulation of the expression of endothelial–leukocyte adhesion molecules: by producing oxidative damage to macromolecules of the endothelium and by the activation of NF-κB (30, 31). The adhesion molecules E-selectin, VCAM-1, and ICAM-1 contain sequences in their promoter regions that are recognized by NF-κB (30, 31). Molecular
Fig. 7. (A) The effect of allicin on concanavalin A (Con A)-induced inducible nitric oxide synthase (iNOS) expression was determined by western blot analysis in liver homogenates 24 h after Con A administration from the following treatment groups: (a) no treatment (lane 1), (b) allicin only (lane 2), (c) Con A only (lanes 3 and 4), (d) Con A + allicin (lanes 5 and 6). Con A administration (c, lanes 3 and 4) induced the expression of iNOS compared with the two control groups (a and b, lanes 1 and 2). This expression was significantly reduced by allicin (d, lanes 5 and 6). (B) The ratio of densitometric results of iNOS expressed as percentage of controls. Mean ± SD, n = 4, **P < 0.01 compared with Con A.

genetic analysis has demonstrated the role of NF-κB in cytokine-induced endothelial expression of adhesion molecules and that inhibition of NF-κB can abolish the expression of ICAM-1 and VCAM-1. Since TNF-α and iNOS signals NF-κB activation through a pathway that involves reactive oxygen intermediates, allicin pretreatment might enhance antioxidant defence mechanisms and thereby suppress NF-κB activation, which in turn, will lead to inhibition of ICAM-1 and VCAM-1 expression on the endothelium.

Pharmaceutical preparations of garlic-related compounds are already used therapeutically to decrease serum lipid levels (3). Further investigation that will include safety and dose-finding studies are necessary to determine whether these compounds should also be considered to inhibit immune-mediated pathological conditions in the liver.

References


