Inhibition of Carcinogen-Induced DNA Damage in Rat Liver and Colon by Garlic Powders With Varying Alliin Content

Varsha Singh, Christine Belloir, Marie-Hélène Siess, and Anne-Marie Le Bon

Abstract: The present study was designed to investigate the protective efficiency of three garlic powders, obtained from bulbs grown in soils with different levels of sulfur fertilization, against DNA damage. Increasing fertilization of soil resulted in an increased alliin content of the powders. Garlic powders were administered to rats for 2 weeks (5% of the diet) and their antigenotoxic effects were examined in the liver and the colon using the comet assay. Consumption of the different garlic powders induced a 35–60% reduction in DNA damage induced by N-nitrosodimethylamine (NDMA) in rat liver. Increased alliin content of the garlic powder was associated strongly with a proportional decrease in NDMA-induced DNA alteration. DNA damage induced by aflatoxin B1 in the liver or by 1,2-dimethylhydrazine in the colon were also decreased strongly by the three garlic powders but these decreases were not correlated to the alliin content of the garlic powders. Feeding garlic powders did not modify the genotoxic activity of the direct-acting carcinogen methyl-nitrosourea in the colon. Part of our results supports evidence that fertilization can have an impact on the protective capacity of garlic bulbs.

Introduction

Garlic (Allium sativum) is consumed worldwide and a variety of beneficial properties (including antibacterial, antithrombotic, anticarcinogenic, antiatherosclerotic, and hypolipidemic activities) have been reported since ancient times. Garlic contains characteristic water- and lipid-soluble organosulfur compounds (OSC), which are responsible for the pungent smell and probably contribute to its beneficial health properties. The intact garlic bulb is odorless, but when it is cut or crushed, alliin (S-allylcysteine sulfoxide), the most abundant sulfur compound in garlic, is converted into allicin (diallyl thiosulfinate) by the catalytic action of a specific enzyme, alliinase (1). Allicin is an unstable compound and readily degrades via several pathways to a variety of compounds, mainly diallyl mono-, di-, and oligosulfides, vinylthiins, and ajoenes (2). In addition to fresh whole cloves, many types of processed garlic formulations are available commercially, such as powdered dry garlic, steam-distilled oil, oil-macerated products, or aged alcoholic extracts. These preparations differ considerably from one another in the composition of OSCs as a result of chemical and enzymatic changes that take place during processing (3). The OSC content of garlic bulbs also varies according to environmental factors, degree of maturation, and storage conditions. The cultural conditions may modify the composition of garlic significantly. The amount of sulfur in garlic bulbs grown in sand culture in a greenhouse is influenced by the level of sulfate in the nutrient medium (4). Therefore, manipulating growing conditions to increase the OSC content of garlic could be a key for optimizing the benefits of garlic.

The evidence of an anticarcinogenic role for garlic comes from both epidemiological and experimental investigations. The consumption of garlic has been associated with a reduction in cancer risk in various parts of the world (5). A number of studies have evaluated the effects of different garlic preparations on chemical carcinogenesis in animal experiments (6). Most of these studies showed an inhibition or a reduction of tumor formation in several tissues when garlic preparations were administered before or simultaneously with the carcinogens. Several garlic preparations were also shown to protect DNA against alterations induced by various chemicals in rodent tissues (7–9). These chemopreventive properties have been attributed to the OSCs found in garlic. However, in most of these cases, the garlic preparations were not characterized chemically. It is therefore difficult to compare the properties of the different garlic preparations studied so far.

The objective of the present study was to investigate the protective efficiency of well-characterized garlic powders, containing varying levels of alliin, against DNA damage caused by chemical carcinogens in the rat. For this purpose, we used garlic bulbs grown in soils with different levels of sulfur fertilization, and we characterized the composition of garlic powders derived from these bulbs, especially the level of alliin. The different powders were administered to rats for 2 weeks and their antigenotoxic effects were investigated in the liver and colon using the comet assay. In previous studies, we demonstrated that garlic powder consumption decreased...
cytochrome P450 (CYP) 2E1 and increased CYP1A2, uridine diphosphate–glucuronosyl transferase (UGT), and glutathione-S-transferase (GST) activities in the liver of rats (10,11). This suggested that garlic powder could have a chemopreventive effect against the toxicity of indirect-acting carcinogens, which are biotransformed by these enzymes. To test this hypothesis, we used aflatoxin B1 (AFB1) and N-nitrosodimethylamine (NDMA) to induce DNA damage in the liver and 1,2-dimethylhydrazine (DMH) to induce similar effects in the colon. We also searched for any protective effect against the direct-acting carcinogen methylnitrosourea (MNU) in the colon.

Materials and Methods

Chemicals

Minimum essential medium (MEM) with glutamax® Earle salt and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, phosphate-buffered saline (PBS) without calcium and magnesium, and trypan blue solution were obtained from Invitrogen (Cergy Pontoise, France). Collagenase type IV, dimethylsulfoxide (DMSO), Na2 ethylenediaminetetraacetic acid (Na2EDTA), Triton X-100, propidium iodide, sodium sarcosinate, low melting point (LMP) agarose, tri-hydroxyaminomethane (Tris) buffer, AFB1, NDMA, MNU, and DMH were purchased from Sigma-Aldrich (La Verpillière, France). Normal melting point agarose was from Coger Promega (Charbonnières les Bains, France). NDMA, MNU, and DMH were dissolved in sterile NaCl (0.9%) just before use. AFB1 was diluted in DMSO. Other chemicals were of the highest quality available.

Plant Materials, Cultivation, and Preparation of Garlic Powders

Garlic (A. sativum) was produced the same year (2001) in a field trial carried out in Crest (Drôme, France). Certified seed material (variety Printanor) was supplied by the Institut National de la Recherche Agronomique d’Avignon (France). Seeds were planted early in the year (January) and harvested 6 months later. Sulfur fertilization was provided by dehydrated CaSO4, which was applied twice before bulb formation (12). Three levels of CaSO4 were used: 0, 100, and 200 kg/ha. Four replicates of 100 plants were made for each level of CaSO4. Bulbs were harvested when considered mature (juice above 30° Brix) and air-dried. When completely dry (3–4 weeks later), the bulbs were processed as described previously (10) to obtain a fine powder (<25 µm particles).

Analysis of Sulfur Compounds in the Powder

Aliquots of the different garlic powders were analyzed by high-performance liquid chromatography as described previously (10,13). Briefly, after extraction with acidified methanol/water (80/20, vol/vol), compounds were separated on a Hypurity Elite C18 column Thermo Quest at 38°C (Thermo Hypersil, Keystone, Bellefonte, PA) and detected at 208 nm using a diode array detector (Waters, Milford, MA). Sulfur compounds were characterized by comparison of their retention times and their spectra with standard compounds synthesized previously (13).

Animals and Treatments

Male 4-week-old specific pathogen free (SPF) Wistar rats were purchased from Janvier (Le Genest St Isle, France). They were housed in individual stainless wire cages, in a room maintained at 21°C, with constant humidity and a 12-h light–dark cycle. Rats were maintained in accordance with the French Ministry of Agriculture Guidelines for care and use of laboratories animals. They were fed a purified diet (Table 1). The composition of mineral and vitamin mixtures was described previously (14). Water was added to the diet in the ratio of 50 g water/100 g dry matter.

Two separate studies were conducted. First, to study the effects of garlic on liver, 60 rats were fed the control diet for 1 week for acclimatization and were then randomly allocated to four groups of 15 rats each. The control group (Group C) was given the purified diet throughout the experiment. Experimental groups, designated as S0, S100, and S200, were given the same diet for 2 weeks with the addition of 5% of garlic powders derived from garlic bulbs grown on soils fertilized with 0, 100, or 200 SO4 kg/ha, respectively. The garlic powders were incorporated into the diet at the expense of sucrose and casein (Table 1). At the end of the feeding period, five rats from each group were injected intraperitoneally with a single dose of AFB1 (2 mg/kg body weight) or NDMA (0.4 mg/kg body weight) and killed 4 h later. The liver was removed and rinsed three times with cold sterile PBS. A piece of ~2 g was placed in PBS (1 ml/g) and minced briefly with fine scissors. The sample was filtered through three layers of sterilized gauze. Ten microliters of the resulting cell suspension (~10,000 cells) was used to perform the comet assay.

In the second experiment, the goal of which was to evaluate the effects of garlic in the colon, 60 rats were randomly allocated to four groups of 15 rats each using the same feeding regimen as the first study. At the end of the feeding period, five animals in each group received a single dose of MNU (20

<table>
<thead>
<tr>
<th>Ingredients (g/100 g diet)</th>
<th>Control Diet</th>
<th>Garlic Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td>Starch</td>
<td>46</td>
<td>46</td>
</tr>
<tr>
<td>Sucrose</td>
<td>23</td>
<td>19</td>
</tr>
<tr>
<td>Cellulose</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Mineral mixture</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Garlic powder</td>
<td>—</td>
<td>5</td>
</tr>
<tr>
<td>Corn oil</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
mg/kg body weight, gavage) or DMH (200 mg/kg body weight, intraperitoneally), 3 and 24 h prior to sacrifice, respectively. A segment of approximately 10 cm length was removed in the distal colon and rinsed carefully with cold Merchant solution (i.e., PBS plus 0.53 mM Na$_2$EDTA). The segment was opened longitudinally, cut into small pieces, and incubated in 2 ml MEM containing collagenase (0.5 mg/ml) for 20 min at 37°C with gentle stirring (150 rpm). The resulting suspension was filtered through a nylon membrane (80 µm pore diameter) then centrifuged at 170 g for 1 min at 4°C. The cell pellet was suspended in 100 µl PBS and 20 µl of the resulting cell suspension was used to perform the comet assay. Cell viability was checked using the trypan blue exclusion assay and was found to be greater than 75%.

Comet Assay

The alkaline version of the comet assay was carried out according to the procedure of Singh et al. (15), with slight modifications. Briefly, cell suspensions were mixed with 75 µl 0.5% LMP agarose and placed on microscope slides (Kimble Kontes, Baillet-en-France, France) that were already precoated with 1% normal melting point agarose. After solidification, slides were covered with a third layer of 0.5% LMP agarose (75 µl). Slides were immersed into a lysing solution (1% sodium sarcosinate, 2.5 M NaCl, 100 mM Na$_2$EDTA, 10 mM Tris-HCL, pH10, 1% TritonX-100, and 10% DMSO) for 60 min at 4°C. Then all slides were placed in an electrophoretic tank containing the electrophoresis buffer. DNA unwinding was allowed to proceed for 40 min at room temperature and electrophoresis was conducted for the next 20 min at 25 V (300 mA) using a compact power supply (ST600, Apelex, Paris, France). After electrophoresis, the slides were rinsed with 0.4 M Tris (pH 7.5) and fixed by dipping them for 1 min in 96% ethanol. They were allowed to dry at air temperature until staining.

Each slide was stained by rehydrating the sample on the slide with 75 µl propidium iodide solution (2 µg/ml water) and covering it with a coverslip. Slides were examined at ×200 magnification using a fluorescence microscope (excitation filter 515 nm, barrier filter 560 nm; Nikon E600, Champigny/Marne, France). Image analysis was performed using the software Komet IV (Kinetic Imaging, Liverpool, United Kingdom) on 100 randomly selected cells (50 cells from each of two replicate slides). The extent of DNA damage was quantified by the olive tail moment (OTM), which is defined as the product of the mean distance of DNA migration in the tail and the percentage of DNA in the comet tail (16). Data are presented as the mean ± standard error of the mean of the OTM values (n = 5 rats).

Statistical Analysis

The data were submitted to an analysis of variance followed by a Dunnett’s test to assess the difference between OSC-treated groups and untreated groups and a Newman Keuls’s test to perform comparisons between groups (17–19). Results were considered to be significant if a P value ≤0.05 was recorded. Correlation analysis (Pearson’s correlation coefficient) was used to test the relationship between the alliin content of the powder and the DNA damage level. The statistical analyses were carried out using the StatBoxPro version 5 software (Grimmersoft, Paris, France).

Results

Sulfur Analysis of Garlic Powders

The following compounds were identified in the garlic powders: alliin, S-allyl-L-cysteine, γ-glutamyl-phenylalanine and two precursors of alliin, γ-glutamyl-S-allyl-L-cysteine and γ-glutamyl-S-(trans-1-propenyl)-L-cysteine. Alliin was identified as the main component and the levels of alliin were quantified in the different powders (Table 2). A strong relationship was found between the level of sulfate fertilization and the alliin content of the garlic powders ($r = 0.915$).

DNA Damage

Figure 1 shows the impact of garlic powders on DNA damage induced by AFB1 and NDMA in the liver of rats. The consumption of the garlic powders S0, S100, and S200 decreased the level of NDMA-induced DNA damage by 35, 51, and 60%, respectively. Correlation analysis showed that the DNA damage level in these groups was inversely correlated to the alliin content of the garlic powders (Table 3). The inhibitory effects of S100 and S200 powders were significantly higher than that of the S0 powder (Newman-Keul’s test). DNA alterations induced by AFB1 were significantly decreased by garlic powder consumption (36–48% reduction). An increasing trend in DNA damage across S0–S200 groups in rats treated with AFB1 was observed. Nevertheless, this effect was not statistically different ($r = 0.174$, $P = 0.275$; Table 3).

In the colon (Fig. 2), all the treatments induced a strong reduction of DNA alterations in rats treated with DMH (51–64% inhibition compared with the DMH-injected control rats). The decrease in DMH-induced damage was not correlated to the alliin content of the garlic powders (Table 2).

<table>
<thead>
<tr>
<th>Garlic Powder</th>
<th>Level of S Fertilization (kg CaSO4/ha)</th>
<th>Alliin Concentration (nmol/mg garlic powder)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0</td>
<td>0</td>
<td>57.8 ± 4.8†</td>
</tr>
<tr>
<td>S100</td>
<td>100</td>
<td>75.2 ± 2.7†</td>
</tr>
<tr>
<td>S200</td>
<td>200</td>
<td>90.2 ± 4.4†</td>
</tr>
</tbody>
</table>

$a$: Values are means ± standard error of the mean of two or three replicates. Means with different superscript symbols (†, ‡, §) are significantly different (Newman Keuls’s test, $P < 0.05$).
3). Consumption of garlic powders did not modify the genotoxic activity of MNU in the colon.

The extent of DNA damage in the liver and colon of rats receiving only garlic powders was the same as in the control rats (Figs. 1 and 2).

**Discussion**

The present study evaluated the effect of three garlic powders containing increasing alliin levels on the genotoxicity of nitrosamines in rat tissues. We found that garlic powder ingestion caused significant reduction of DNA damage in the liver and colon of rats treated by NDMA and DMH, respectively. Interestingly, ingestion of garlic powder containing increasing concentrations of alliin resulted in an increased reduction of DNA damage induced by NDMA. To our knowledge, this is the first evidence that dehydrated garlic has an antigenotoxic activity toward nitrosamines in the colon. In the liver, our findings are consistent with previous studies showing that ingestion of crushed garlic depressed formation of O6-methylguanine in liver DNA of rats injected with NDMA (20,21).

Induction of DNA alteration by NDMA and DMH depends on their metabolic activation to form a diazonium ion capable of forming DNA adducts (22–24). Liver CYP2E1 is thought to be the main CYP involved in the activation of these carcinogens. In a previous study, we showed that feeding rats with garlic powder induced a reduction of hepatic CYP2E1 activity (10). It can therefore be assumed that the decrease in hepatic CYP2E1 activity affects the activation of NDMA and DMH, which results in a reduction of the genotoxic activity of these compounds in rat tissues.

However, other enzymes, such as alcohol dehydrogenase, choline dehydrogenase, and prostaglandin synthase, have been reported to be involved in DMH metabolism in the colon and in other extrahepatic organs (25–27). Thus, the decrease of DMH genotoxicity can also result from modification of the activity of these enzymes by garlic. Involvement of oxygen radicals in DMH genotoxicity also has been suggested (28). Given that garlic preparations have been reported to have antioxidative properties (29,30), these proper-

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**Table 3.** Correlation of the Level of DNA Damage With the Level of Alliin

<table>
<thead>
<tr>
<th>Compound</th>
<th>Group</th>
<th>Correlation Coefficient</th>
<th>Associated $P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDMA</td>
<td>With genotoxic</td>
<td>–0.898</td>
<td>$&lt;1 \times 10^{-4b}$</td>
</tr>
<tr>
<td></td>
<td>Without genotoxic</td>
<td>–0.785</td>
<td>$4.46 \times 10^{-4b}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–0.609</td>
<td>0.003$^b$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.174</td>
<td>0.275</td>
</tr>
<tr>
<td>AFB1</td>
<td>With genotoxic</td>
<td>–0.753</td>
<td>$9.79 \times 10^{-5b}$</td>
</tr>
<tr>
<td></td>
<td>Without genotoxic</td>
<td>–0.119</td>
<td>0.342</td>
</tr>
<tr>
<td>DMH</td>
<td>With genotoxic</td>
<td>0.211</td>
<td>0.193</td>
</tr>
<tr>
<td></td>
<td>Without genotoxic</td>
<td>–0.117</td>
<td>0.345</td>
</tr>
<tr>
<td>MNU</td>
<td>With genotoxic</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Without genotoxic</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$: Abbreviations are as follows: NDMA, N-nitrosodimethylamine; AFB1, aflatoxin B1; DMH, 1,2-dimethylhydrazine; MNU, methylnitrosourea. Correlation analysis (Pearson’s correlation analysis) was performed either with or without the group treated with the genotoxic compound alone included in the calculation.

$^b$: Correlation was considered to be statistically significant when $P < 0.05$. 

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ties might also contribute to the chemopreventive action of garlic toward DMH genotoxicity.

We also showed that garlic powder protects rats against liver DNA damage induced by another indirect-acting compound, AFB1. An apparent increasing trend in DNA damage across S0–S200 groups in rats treated with AFB1 was observed but statistical analysis (Newman-Keul's test and correlation analysis) indicated that this effect was not significantly different. The clinical significance of this trend remains to be determined but it cannot be explained by a direct or indirect effect of garlic powders on AFB1-induced DNA damage. On the one hand, garlic powders did not induce significant DNA damage by themselves in the liver (Fig. 1). On the other hand, we have shown in previous studies that garlic powders prepared according the same procedure failed to increase the activity and expression of CYP3A, which is involved in AFB1 activation (10,11). Conversely, consumption of these garlic extracts enhanced the activity of rat hepatic enzymes (CYP1A, GST, and UGT), which play key roles in the detoxification of AFB1; significant correlations between induction of these enzymes and reduction in the number of hepatic foci initiated by AFB1 have been found (10,11). Therefore, we believe that the garlic powders S0, S100, and S200 reduced AFB1-induced DNA damage at the same extent in the present study. This is in agreement with the result of a previous carcinogenesis experimentation from our group, which showed that S0 and S200 powders, issued from the same batches as those used in the present study, reduced the initiation stage of hepatocarcinogenesis AFB1-induced and enhanced liver phase II enzymes at the same level (11).

Garlic consumption did not modify MNU genotoxicity in the colon. This observation suggests that constituents of garlic powder do not act as scavengers of this direct-acting compound in this organ. Diallyl sulfide (DAS), a sulfur compound present in garlic oil, also failed to inhibit MNU-induced nuclear aberrations in colon of mouse (31). However, dietary garlic supplementation was reported to reduce MNU-induced methylation of DNA in rat mammary gland (32). Treatment of rats with an aqueous garlic extract was also found to reduce N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced clastogenicity in bone marrow (33). The reason for these opposite findings in studies using direct-acting compounds is unclear. Differences in the target organ or the type of garlic preparations could explain these discrepancies.

In the present study, we explored the effects of different garlic powders obtained from bulbs grown in soils with different levels of sulfur fertilization. The alliin content of garlic powders was found to be strongly associated with the sulfur supply, which confirms that it is possible to modify the composition of garlic bulbs by environmental factors. The alliin level of garlic powders was found to be inversely associated with the decrease of NDMA-induced DNA damage in liver. Conversely, increasing the level of sulfur fertilization, and therefore the alliin content of garlic powder, had no influence on the level of the protection afforded by garlic consumption in rats treated with AFB1 and DMH. The reason for such a selective effect is unknown.

There is some evidence that alliin is not the biologically active compound of garlic powder. Alliin was reported to be ineffective in blocking DNA adduct formation induced by dimethylbenzanthracene in rat mammary tissue (34). In addition, alliin has been shown to be converted rapidly to allicin when garlic powder becomes wet (35). The allicin-releasing capacity of garlic powders used in this study has been checked, as described previously (13). Allicin is very unstable and decomposes rapidly to other sulfur compounds, notably to diallyl disulfide (DADS), which could be one of the active metabolites of garlic. DADS has been identified as a metabolite of allicin in perfused rat liver (36) and has been
detected in human urine after garlic oil ingestion (37). DADS treatment previously was shown to reduce strongly DNA damage and incidence of preneoplastic foci in liver of rats treated by chemical carcinogens (38,39). Moreover, DADS induced effects on drug metabolizing enzymes qualitatively similar to that produced by garlic consumption (10). Other sulfur compounds present in the powder, such as γ-glutamyl peptides, could also be active compounds.

In conclusion, our data demonstrated that garlic powders were effective against indirect-acting genotoxic compounds such as nitrosamines and AFBl in rat tissues but not against the direct-acting compound MNU. These findings support the hypothesis that garlic compounds act through modulation of drug-metabolizing enzymes. Results obtained in groups treated both with NDMA and garlic powders, associated with those of previous studies (10,11), also provide evidence that fertilization may have an impact on the protective capacity of garlic bulbs. Controlling culture conditions could therefore be a convenient way to enhance the amount of protective compounds in vegetables.

Acknowledgments and Notes

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