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Systemic Production of IFN-α by Garlic (*Allium sativum*) in Humans

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ABSTRACT

The effect of foods on the production of interferon-α (IFN-α) is currently unknown. Garlic (*Allium sativum*) used as a folk medicine is reported to stimulate nitric oxide (NO) production. We investigated the systemic increase of NO due to the ingestion of garlic on the plasma IFN-α level in normal volunteers. Normal volunteers (10 groups, 10 in each group) ate 2 g fresh garlic, and plasma NO and IFN-α levels were determined after 2 and 4 h. The participants were also asked to eat garlic for various periods of time, and plasma NO and IFN-α were similarly assayed. Ingestion of 2 g fresh, but not boiled, garlic was found to increase the basal plasma level of NO from 2.7 ± 0.1 μM to 8.76 ± 0.21 μM at 2 and 4 h, respectively. The basal plasma IFN-α level increased from 9.51 ± 0.26 nM to 46.3 ± 1.2 nM in normal volunteers ($n = 10$) at the same time. The chronic eating of garlic was found to maintain IFN-α at high levels for at least 7 days. The exposure of neutrophils to garlic *in vivo* or *in vitro*, which also stimulated synthesis of NO in these cells, was found to stimulate IFN-α synthesis as measured by the stimulation of IFN-α mRNA synthesis. Thus, consumption of garlic resulted in stimulated synthesis of NO and, in turn, IFN-α in humans, which could be beneficial in viral or proliferative diseases.

INTRODUCTION

Interferons (IFNs) are a family of heterogeneous small protein molecules well known for their antiviral activity (for review, see refs. 1 and 2). The IFNs are produced in cells infected with different viruses and by double-stranded nucleic acids. Although IFNs are usually administered through subcutaneous (s.c.) or intramuscular (i.m.) routes, oral administration of IFNs is also reported to be therapeutically effective.[3]

Garlic (*Allium sativum*) is used as a vegetable in cuisines of many countries. Fresh garlic has been eaten as a folk remedy for the common cold and for the prevention of infectious diseases in various parts of India from antiquity. We have reported before that feeding of fresh garlic to white Swiss mice resulted in an increase in the plasma nitric oxide (NO) level.[4] Because NO is a biologic messenger molecule and has been reported to induce synthesis of anticancer proteins[5] and IFN-α is a well-known anticancer cytokine,[6] we investigated the effect of an increased plasma NO level on the increase in the plasma IFN-α level in normal volunteers. Furthermore, we investigated the role of garlic-induced NO synthesis on the synthesis of IFN-α by exposing human neutrophils to garlic both *in vivo* and *in vitro* by determining the synthesis of IFN-α mRNA in relation to the synthesis of NO in these cells.

MATERIALS AND METHODS

Chemicals

Recombinant human IFN-α (rHuIFN-α), N^G-nitro-L-arginine methyl ester (L-NAME) and goat antirabbit IgG-alkaline phosphatase were from Sigma Chemical Company (St. Louis, MO). ELISA Maxisorp plates were from NUNC (Roskilde, Denmark). All other chemicals used in the study were of analytic grade.

Selection of volunteers

Equal number of both male and female volunteers ($n = 100$) between the ages of 22 and 50 years participated in the study. None of the volunteers at the time of presentation had diabetes mellitus, systemic hypertension, severe infection, or life-threatening cardiovascular conditions. These volunteers were asked not to take any medication, including aspirin, for at least 14

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days before blood donation. The participants were asked to completely avoid all food preparations containing garlic for at least 4 weeks before and during the study.

This study, which involved the participation of normal adult volunteers, was approved by the Internal Review Board of the Institute. All participants signed informed consents.

Collection of garlic

Only fresh and wholesome garlic bulbs were obtained from local vendors. Care was taken to collect garlic bulbs with no visible cut marks or blemishes. Before use, the bulbs were freed of roots and dried skin and thoroughly washed with distilled water to remove dirt and debris. The washed bulbs were subsequently air dried and used immediately.

Ingestion of garlic by volunteers

Each volunteer ate 2 g of fresh garlic (wet weight of 2–3 cloves of garlic, equivalent to 1.06 g of dry weight of the vegetable containing 27.5 mg protein/g dry weight). In certain phases of the study, the volunteers ate 1, 2, 4, or 6 g fresh garlic. They also ate boiled garlic bulbs (2 g), which were prepared by heating fresh bulbs in a boiling waterbath for 10 min. After cooling to ambient temperature (24°C), the garlic bulbs were eaten by the volunteers.

Preparation of garlic extract

Fresh garlic (4 g) was crushed at 4°C in a mortar and pestle. The crushed mash was centrifuged at 10,000*g* at 0°C. The supernatant portion was used as garlic extract, which contained 27 mg protein/mL and was used immediately.

Raising of polyclonal antibody against rHuIFN-α

Polyclonal antibody against pure rHuIFN-α were raised by repeated immunization in New Zealand rabbit as described.[7]

Enzyme-linked immunosorbant assay (ELISA) for IFN

Only plasma levels of IFN-α, not serum IFN-α levels, were studied. ELISA was performed as described.[8] Briefly, IFN-α was incubated with an equal volume of phosphate-buffered saline (PBS) in an assay plate overnight at 4°C. Nonspecific binding was blocked by using 0.5% bovine serum albumin (BSA) in the same buffer. The samples were next washed with PBS containing Tween-20 and subsequently incubated with diluted antibody in PBS (1:500) for 2 h, followed by washing with the same buffer containing Tween-20. The samples were next incubated with diluted goat antirabbit IgG-alkaline phosphatase (1:2000) in PBS, followed by washing. They were then incubated with *p*-nitrophenyl phosphate (1 mg/mL) in carbonate buffer containing 10 mM $MgCl_2$. The development of color was determined at 405 nm. The amount of IFN-α produced in the neutrophils was determined by constructing a standard graph using different amounts of rHuIFN-α. The reproducibility of the ELISA for determination of IFN-α was verified by determining analytic precision by the recovery of the added IFN-α in the assay mixture containing human cell free plasma from blood. Before determination of IFN-α by ELISA, the feasibility of the assay was determined using the immunoblot technique.[9]

Collection of blood samples and preparation of plasma and neutrophils

Blood samples were drawn from volunteers by venipuncture using 19-gauge siliconized needles, collected in plastic vials, anticoagulated by the addition of 9 volumes of blood to 1 volume of 0.13 M sodium citrate, and mixed by gentle inversion.[10] Cell-free plasma was prepared by centrifuging the blood sample at 20,000*g* for 20 min at 0°C. Plasma thus prepared was used for determination of IFN-α in the sample. Neutrophils were prepared from the blood samples using the Ficoll-Hypaque method as described.[11] Neutrophil numbers were determined by light microscopy.

In vitro *translation of IFN-a mRNA*

mRNA was extracted using the Trizol method from neutrophils isolated from blood samples.[12] Briefly, the mRNAs were incubated with the ribosomal preparation, a mixture of amino acids, and ATP as described.[13] After 6 h, the reaction mixture was centrifuged at 10,000*g* at 0°C for 10 min. The supernatant was used for determination of IFN-α by ELISA as described.

Determination of plasma NO level

The NO level in the plasma samples was determined by conversion of oxyhemoglobin to methemoglobin as described[14] and independently verified using the chemiluminescence method.[15] Protein was determined by the method of Lowry et al.[16]

Statistical analysis

The significance (p) of the results was determined using the Student's t-test; $p < 0.0005$ was considered significant.

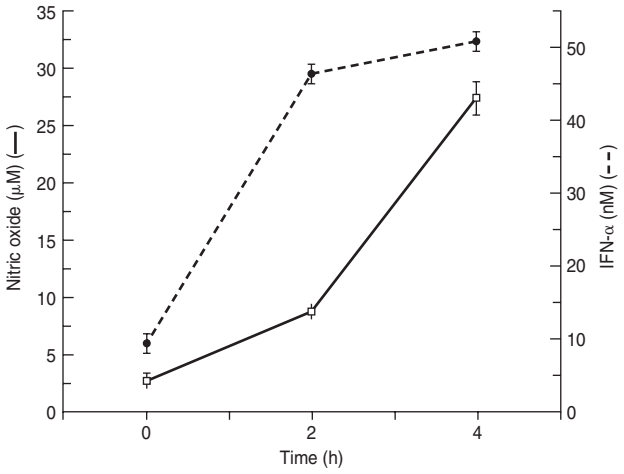

FIG. 1. Plasma IFN-α and NO levels in normal volunteers at different times after eating fresh garlic. Normal volunteers (n = 10, 5 men, 5 women) ate 2 g fresh garlic as described. Plasma levels of NO (solid line) and IFN-α (broken line) were determined at different times, as indicated. Each point represents the mean ± SD of 10 different experiments.

RESULTS

Effect of ingestion of fresh garlic on plasma NO and IFN-α levels

Ingestion of fresh garlic (2 g wet weight) by normal volunteers were found to cause an increase in the basal plasma NO level of 2.7 ± 0.1 μM to 27.37 ± 0.87 μM after 4 h of eating the vegetable. The plasma IFN-α level increased from the basal 9.51 ± 0.26 nM to 50.7 ± 0.16 nM at 4 h (Fig. 1). The serum level of IFN-α has been reported to be 15.6–500 pg/mL,[17] but we have found that the basal plasma level of IFN-α was considerably higher.

In a separate experiment, when different amounts of fresh garlic were ingested by normal volunteers, it was found that 4 h after eating the vegetable, the highest plasma NO level was recorded after eating 2 g of the fresh garlic. The highest plasma IFN-α level was recorded at 4 h when 4 g of garlic was eaten; however, the plasma NO level decreased (Table 1). In contrast, the ingestion of boiled garlic (2 g) did not cause an increase in either the NO or IFN-α level in plasma over the corresponding basal values. The basal levels of plasma NO and IFN-α, which were 2.68 μM and 10.6 nM, respectively, remained essentially unchanged after 4 h (NO = 2.74 μM, IFN-α = 11.6 nM) when the volunteers ate the boiled garlic.

Maintenance of increased plasma IFN-α level by garlic

To determine the length of time the increased plasma IFN-α as a result of eating garlic was maintained *in vivo*, the volunteers ate 2 g fresh garlic once. The plasma NO and IFN-α levels were determined up to 3 days. Although the increased plasma NO level due to garlic ingestion returned to basal level (2.71 μM) after 12 h, the plasma level of IFN-α continued to increase at least up to 48 h. After 48 h, the plasma IFN-α level decreased, but even after 72 h, the plasma cytokine level was higher than that of the basal level at the beginning of the experiment. The increased cytokine level begins to decrease 2 days after ingestion of 2 g fresh garlic (Fig. 2).

Daily eating of 2 g fresh garlic maintained the elevated level of plasma IFN-α at least up to the 7 days tested (Fig. 3). Similar experiments indicated that the increase in the plasma IFN-α level could be sustained by eating garlic every 2 days (data not shown). It was not necessary to eat garlic daily to maintain the increased plasma level of IFN-α.

Table 1. Effect of Consuming Different Amounts of Garlic on Plasma NO and IFN-α Levels in Normal Volunteers[a]

Garlic (g)	*Nitric oxide (μM)*	*IFN-α (nM)*
0	2.71 ± 0.08*	9.51 ± 0.10*
1	6.74 ± 0.12*	12.87 ± 0.10*
2	27.37 ± 0.18*	50.4 ± 0.90*
4	10.01 ± 0.20*	57.52 ± 0.37*

[a]Ten normal volunteers (5 female and 5 male) ate different amounts of fresh garlic as indicated. After 4 h, blood samples were collected by venipuncture, and the plasma NO and IFN-α levels were determined as described in Materials and Methods. Results are the mean ± SD of 10 different experiments.
*$p < 0.005$.

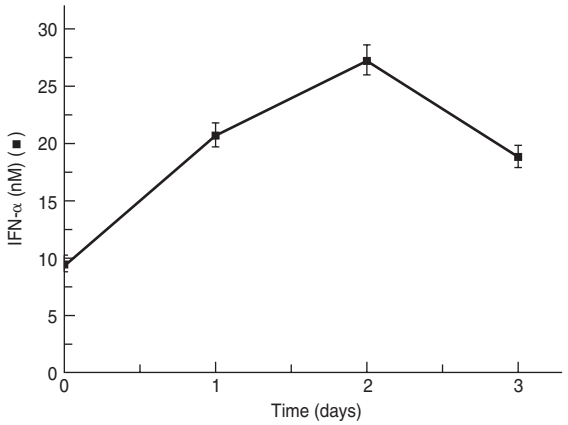

FIG. 2. Effect of ingesting 2 g fresh garlic one time only on the plasma IFN level in normal volunteers at different times after eating. Normal volunteers (Fig. 1) ate 2 g fresh garlic once, and the plasma IFN-α levels were determined at the indicated day after garlic was consumed. Each point represents mean ± SD of 10 different experiments involving 10 different volunteers. The plasma NO level was also determined on the indicated day. The plasma NO level returned to the basal level at 12 h after garlic was eaten; consequently, the plasma NO is not shown.

Synthesis of IFN-α in human neutrophils as a result of in vivo *and* in vitro *exposure of these cells to garlic*

IFN-α is known to be produced in the system because of viral infection in the host. The effect of garlic on the increase in the plasma cytokine could be a consequence of release of the preformed IFN-α from the neutrophils related to previous viral infection through increased NO synthesis. NO could merely be involved in the release process instead of stimulating synthesis of the cytokine. To determine if garlic could actually induce systemic synthesis of IFN-α, volunteers ate 2 g fresh garlic as described, and after 4 h, blood samples were drawn. Plasma NO was determined, and neutrophils were prepared from the blood samples as described.[11] Subsequently, the mRNAs were extracted from neutrophils, and the presence of IFN-α mRNA was determined by *in vitro* translation. The synthesized IFN-α in the *in vitro* reaction mixture was determined by ELISA. We found that either systemic exposure of neutrophils to garlic by ingestion or *in vitro* exposure of neutrophils to garlic extract (by adding 40 μL of fresh garlic extract containing 1.08 mg protein/mL of the neutrophil suspension) caused stimulated synthesis of IFN-α, which was related to the increase in NO synthesis (Table 2). Furthermore, we found that addition of L-NAME, a competitive inhibitor of NO synthesis,[18] inhibited both NO and IFN-α synthesis in these cells (Table 2).

DISCUSSION

Although viral infection is well known to induce IFNs systemically, our results describe for the first time that fresh gar-

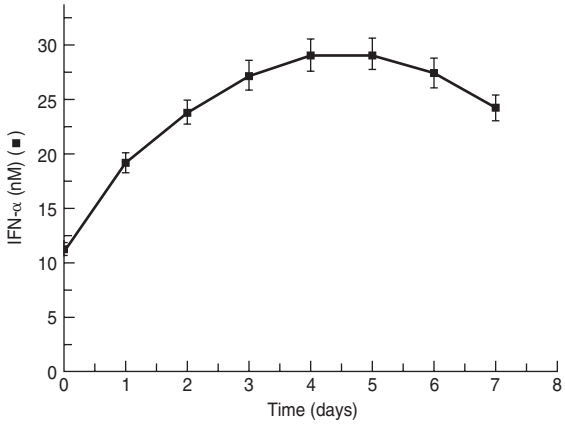

FIG. 3. Effect of eating garlic daily on the plasma IFN-α level in normal volunteers. Ten normal volunteers ate fresh garlic (2 g) every day for 7 days. At the end of every 24 h, the plasma IFN-α level was determined. Each point represents mean ± SD of 10 different experiment using 10 different volunteers (100 people) comprising equal numbers of male and female participants.

lic, a nonviral agent, when ingested in a small quantity (2 g) can increase plasma IFN-α levels within 60 min (Fig. 1). Furthermore, our results demonstrated that it was synthesis of NO by the garlic, not the garlic itself, that was responsible for the stimulated synthesis of IFN-α in neutrophils through increased synthesis of IFN α-mRNA.

Garlic is used for different medicinal purposes ranging from cardiovascular diseases to diabetes and has been reported to enhance immunocompetence.[19,20] It can also suppress inflammatory cytokines.[21] The mechanisms involved in these effects of garlic, however, remain obscure. Our results demonstrate that ingestion of fresh garlic was capable of stimulating IFN-α through the production of NO in humans. NO, a biological messenger molecule,[22] has various regulatory effects on diverse physiologic and pathologic events.[23,24] Our results demonstrate that this messenger is capable of stimulating synthesis of IFN-α, and that once increased synthesis of the cytokine was initiated by NO, the synthetic process continued even in the absence of continued synthesis of NO in the system. Although eating fresh garlic caused increased production of systemic IFN-α, it is not known at present if eating the vegetable would also induce other IFNs (IFN-β or IFN-γ). It could be argued that stimulation of IFN-α by garlic was not due to the garlic itself but due to the presence of virus in the vegetable. However, addition of pure NO solution in 0.9% NaCl[25] to the neutrophil suspension, instead of garlic extract, stimulated IFN-α synthesis in these cells to 4.5 nM (N. Sinha et al., unpublished observations). This effect of NO indicated that the presence of virus in the vegetable is not likely to be the stimulator of IFN-α synthesis.

Consumption of fresh garlic everyday was found to sustain the level of plasma IFN-α. After the fifth day, however, there was a decrease in the plasma cytokine. Furthermore, it was not necessary to eat garlic daily to keep the plasma IFN-α at a high level. Dietary inclusion of 2 g of fresh garlic every 48–72 h was adequate for the maintenance of plasma IFN-α at a significant level.

Our results also demonstrate that the increase in IFN-α in the system was related to newly synthesized not preformed IFN-α

TABLE 2. EFFECT OF GARLIC ON NO AND IFN-α PRODUCTION *IN VIVO* AND *IN VITRO* IN NEUTROPHILS[a]

Cell suspension (10^6/mL)	*Addition*	*Nitric oxide (μM)*	p	*IFN-α (nM)*	p
Control (Volunteers did not eat garlic)	None	2.66 ± 0.2156*,[b]	<0.0005*	19.38 ± 0.26***	<0.0001***
Volunteers ate garlic	None	19.04 ± 0.1*		28.72 ± 0.191***	
Cells (from normal volunteer)	Garlic extract (1.08 mg/mL /10^6 cells) + L-NAME (10 M)	0.94 ± 0.026**		0	
Cells (from normal volunteer)	Garlic extract (1.08 mg/mL /10^6 cells)	3.08 ± 0.205**	<0.0001**	39.7 ± 1.950****	<0.0001****
Cells (from normal volunteer)	None	2.60 ± 0.192**		19.3 ± 0.140****	

[a]*In vivo* experiments. Volunteers (n = 10, 5 men, 5 women) ate 2 g fresh garlic. After 4 h, the plasma NO was determined, and neutrophils were prepared from the blood samples of the volunteers. In control experiments with equal numbers of volunteers who did not eat fresh garlic, neutrophils were similarly prepared, and plasma NO was determined. mRNA from the neutrophils from both groups and the presence of mRNA for IFN-α were determined by *in vitro* translation of the mRNA as described in Materials and Methods. The production of IFN-α in the assay mixture was determined by ELISA of the cytokine. *In vitro* experiments. Neutrophils were prepared from equal numbers of male and female volunteers. In control experiments, the neutrophils were not treated with garlic extract. After 4 h of incubation at 30°C, the formation of both NO and mRNA of IFN-α was determined as described. Each result represents the mean ± SD of five different experiments using five different normal volunteers each in triplicate (3 male, 2 female) for a total of 25 individuals.

[b]The number of asterisks represents the p value of the respective pertaining data.

(Table 2). The addition of NAME, an inhibitor of NO synthesis from L-arginine (substrate) *in vitro* caused complete inhibition of both NO and IFN-α synthesis in neutrophils, indicating an essential role of NO in garlic-induced IFN-α synthesis.

As noted, the stimulated production of NO and, in turn, IFN-α by garlic was mediated by some heat labile factor present in the fresh garlic. These results suggest that the active principle in the fresh garlic might be proteinous in nature and mediated through synthesis of NO. We came to this conclusion because we have purified a protein from fresh garlic that was found to be a potent stimulator of NO synthesis.[26] This protein, called "allimin" (MW.4012.4Da), when added to the neutrophil suspension *in vitro*, was found to stimulate the synthesis of both NO and IFN-α (N. Sinha et al., unpublished observations). As NAME inhibited both NO and IFN-α in neutrophils, it is not the garlic itself but the NO induced by the vegetable that was responsible for synthesis of IFN-α. However, it is not known if the same protein is responsible for increased IFN-α production *in vivo*.

IFN-α is used therapeutically in various viral and neoplastic diseases. Consequently, if the effect of fresh garlic on IFN-α could be reproduced in people with viral or proliferative disease, consumption of fresh garlic might be beneficial for such patients. A simple calculation on the systemic increase in the plasma IFN-α level in normal volunteers as a result of eating 2 g fresh garlic was determined to be approximately 300,000 IU (based on 11.1 μg/mL IFN-α = 3×10^6 units). This could be in a useful range as a supplement to the therapeutic doses of the cytokine used in several viral diseases.

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