Iranian J Publ Health, Vol. 40, No.4, 2011, pp.105-111

Original Article

The Effect of Aqueous Garlic Extract on Interleukin-12 and 10 Levels in *Leishmania major* (MRHO/IR/75/ER) Infected Macrophages

MJ Gharavi[1], ë M Nobakht[2,3], S Khademvatan[4,5], F Fani[6], M Bakhshayesh[7], M Roozbehani[8]

[1]*Dept. of Medical Parasitology, Tehran University of Medical Sciences, Tehran, Iran*
[2]*Dept. of Histology and Neuroscience, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran*
[3]*Anti-Microbial Resistance Research Center, Tehran University of Medical Sciences, Tehran, Iran*
[4]*Dept. of Parasitology, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran*
[5]*Cellular and Molecular Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran*
[6]*Dept. of Pharmacology, Islamic Azad University, Tehran, Iran*
[7]*Cellular and Molecular Research Center, Tehran University of Medical Sciences, Tehran, Iran*
[8]*Faculty of Allied Medicine, Tehran University of Medical Sciences, Tehran, Iran*

(Received 18 Feb 2011; accepted 19 Nov 2011)

Abstract

Background: The aim of the present study was to investigate the immunomodulation effects of aqueous garlic extract (AGE) in the cultured macrophages infected by *Leishmania major*.

Methods: After J774 macrophages proliferation in RPMI1640 and incubation with *Leishmania* for 72 hours, AGE was added in doses of 9.25, 18.5, 37, 74 and 148 mg/ml for 18, 24 and 48 hours and cell culture supernatants were harvested. The *Leishmania* infected J774 cells to assess the cell viability was examined using trypan blue and methylthiazol tetrazolium assay (MTT). An enzyme-linked immunosorbent assay (ELISA) was performed on cell culture supernatants for measurement of interleukin IL-10 and IL-12.

Results: Dose of 37 mg/ml for 48 hours of garlic extract was the most potent dose for activation of amastigotes infected macrophages. In addition, AGE increased the level of IL-12 in *Leishmania* infected cell lines significantly.

Conclusions: AGE treated cell is effective against parasitic pathogens, and AGE induced IL-12 differentially affected the immune response to invading *Leishmania* parasites.

Keywords: *Leishmania major*, Aqueous garlic extract, IL-10, IL-12, Macrophage, Iran

Introduction

Leishmaniasis is a parasitic disease with different clinical manifestations includes the visceral, mucosa or cutaneous leishmaniasis (1). Members of the *Leishmania* genus are obligate intracellular parasites that replicate in the macrophage. Immunological regulation of host responses to *Leishmania* has been investigated in many animals' models (2). In the *L. major* mouse model macrophages, dendritic cells, NK cells, $CD4^+$ Th1 cells, $CD8^+$T cells, IL-12, IFN-γ, and inducible nitric oxide synthase (iNOS) were defined as the key components of the immune system that contribute to the control of the parasites in vivo (3-5).

*Corresponding Address: Fax: (+2188622533) E-mail: nobakht @yahoo.com

Garlic (*Allium sativum*) is one of the oldest plants used as a medicine; it has been considered a valuable healing agent by many different cultures for thousands of years. Sulfur Compounds of the plant, such as allicin, diallyl trisulphide and ajoene can reduce the development of different protozoan parasites including *Giardia lamblia*, *Leishmania major*, *Leptomonas colosoma*, *Crithidia fasciculata*, *Cryptosporidium baileyi*, *Tetratrichomonas gallinarum*, *Histomonas meleagridis*, *Plasmodium berghei*, *Trypanosoma spp* (6).

Chemokines play an important role in the proper development and functional aspects of macrophage. Macrophages from the heterozygous or wild- type mice were very efficient in killing the *Leishmania* parasites following activation by IFN-γ, ILs and NO. AGE-induced stimulated cells were able to control the infection by *L. major*. IL-12 secreted from the macrophage was uniformly highly susceptible to response to the infection and heterozygous mice. These results provided compelling evidence that NO is an important effectors molecule for the killing of intracellular parasites. In addition, studies with specific gene-deficient mice have indicated that impaired IL-12 responsiveness during *L. amazonensis* infection is mediated by an IL-4-independent mechanism (7-8). IL-10 has been previously implicated in disease progression and long-term persistence of *Leishmania* in both human and experimental animal infections (9-10). IL-10 was initially identified as a product of Th2 cells, inhibiting Th1-cell proliferation, development, and function (11). It is also synthesized by a variety of other cells, including macrophages, monocytes, keratinocytes, dendritic cells, and mast cells (12). IL-10 can inhibit the production of several proinflammatory cytokines, including tumor necrosis factor alpha (TNF-α) and NO, in monocytes and macrophages (13).

The aim of this study was to investigate the effect of aqueous garlic extract (AGE) on cytokine secretion of *L. major*- infected macrophages at the different time points.

Materials and Methods

Preparation of garlic extract

Garlic bulbs were extracted by a modification of the procedure of Fromtling and Bulmer (14-15). The garlic cloves were agitated with 200 ml of sterile distilled water on a shaker for 48 hours in 37 ºC. This mixture was centrifuged at 2300 rpm for 25 min. Then, the supernatant was filtered through Whatman no.1 filter paper (Whatman Corp., Bedford and Mass). The supernatant was sterilized by passing through a 0.2 µm Nalgene filter (Nalgene Labware Div, Nalge/Sybron Corp., Rochester, N.Y.). Samples were dried in a 56 °C oven. The sterile lyophilized extract was kept frozen at -80 °C until used.

J774 Cell line

A BALB/c-derived macrophage-like cell line (J774) was grown in Dulbecco's modified minimum essential medium (DMEM) containing heat inactivated FCS 10%. All media were supplemented with penicillin 100 U/ml, streptomycin 100 µg/ml and amphotericin B 250 ng/ml.

Promastigote

For establishing experimental infection, *L. major* standard strain promastigotes (MRHO/IR/75/ER) was kindly provided by Dr. Mohebali (Tehran University of Medical Sciences,). Briefly 5×10^5 cells/ml *L. major* promastigotes, were cultured in $RPMI_{1640}$ medium (pH 7.2, containing 25 mM HEPES) supplemented with 10% heat inactivated fetal bovine serum and antibiotics at 24 °C for 96 h and subcultured at cell densities of 2×10^7 to 2.5×10^7 cells/ml.

In vitro Leishmania major proliferation assay

Promastigotes of *L. major* were cultured in medium RPMI 1640 containing 10% fetal calf serum for 72 h at 37 ºC. The proliferation of promastigotes was evaluated by counting them every 24 h in a hemocytometer.

In vitro assay of *L. major* growth in macrophage

Promastigotes in the stationary phase of growth were used to infect cultures of J774 cell line at a final ratio of 5 parasites per macrophage. Promastigotes were pelleted at 1000 rpm in a rotator for 10 min at room temperature and then resuspended in RPMI 1640 containing 20% FCS at a concentration of 10^6 parasite/ml. Parasites were washed with RPMI 1640 and immediately prior to addition of parasites, the macrophages were washed with medium. To initiate infection, promastigotes were added to 1 $\times 10^6$ macrophages. After addition of parasites, the macrophages were incubated at 33 ºC in 5% CO2. Infection was allowed to proceed for 24 h and then unphagocytosed parasites were removed by washing with medium, and cells resuspended in RPMI 1640/10% FCS, for 72 h at 37 °C. Macrophages were then fixed in methanol and stained with Giemsa stain for determination of intracellular parasite numbers. The mean percentages of survival in treated cultures were calculated on the basis of the number of *Leishmania* in untreated cultures as 100 %.

Analysis of Garlic extract sensitivity by colorimetric MTT assay

Supernatants were collected at 24, 48, and 72 hours after exposure to AGE, pre incubated with different concentrations of garlic treatment in 9.25, 18.5, 37, 74 and 148 mg/ml. All supernatants were then stored at −20 °C until they were assessed for cytokines. MTT assay for cell viability based on formazan formation from MTT was determined as previously described (16-17). The absorbance was measured at 450 nm using ELISA reader (Awarness, Statface 3100). Data were expressed as percentages of the control (untreated cells) and were the mean ± S.D. of three independent measurements.

Cytokine assays

The supernatants fluid were collected from *Leishmania* infected macrophages after 48 h and kept at -20 ºC until use. The IL-10 and IL-12 concentrations in culture supernatants were determined by an enzyme-linked immunosorbent assay (ELISA) specific for IL-10 and IL-12, using commercial ELISA kits (Bender Med Company, CA, USA). Cytokines secreted by Leishmania infected J774 cell line was measured according to the manufacturer's instructions with cytokine production in vitro (18). The plates were read at 450 nm on a Power wave 200 spectrophotometer (Bio-Tek, Winooski, VT). The cytokine concentration in each sample was extrapolated from a standard curve generated from the measured absorbance obtained from recombinant standards supplied.

Statistical analysis

All the experiments were replicated at least three times, and representative results were presented. Comparisons were made between different of groups. The difference between groups was considered to be significant at $P < 0.05$.

Results

Measurement of effective AGE concentration on Leishmania infected- macrophage by MTT assay

The results of MTT assay complied from three experiments in different times 18, 24, and 48 hours are shown in Fig. 1. The viability of the cells was decreased after 48 hours. In the experiments various AGE concentrations of 9.25, 18.5, 37, 74 and 148 mg/ml were applied on *Leishmania* infected macrophage. The result is indicated that 37 mg/ml was the best

concentration for cells lysis during 48 hours (Fig. 2).

Evaluation of the IL-10 cytokine concentrations

IL10 concentration was measured in *Leishmania* infected macrophage exposed with AGE groups and without AGE groups. Results show that the absorbance rate for IL10 in the *Leishmania* infected macrophage without AGE groups (Fig. 3) is not changed. Also the garlic treatment in *Leishmania* infected macrophage with AGE groups (Figure 4), no indicating any changes.

Evaluation of the IL-12 cytokines concentrations

In *Leishmania* infected macrophage with AGE groups and without AGE groups the in vitro rate of amastigotes in macrophage activity is expressed by IL 12. Data presented in Fig. 5 show that the *Leishmania* infected macrophage without AGE groups, there is a significant decrease of the rate of absorbance for IL12. In contrast by garlic treatment in *Leishmania* infected macrophage with AGE groups, data presented in figure 6 show a significant increase of the rate of absorbance.

Results showed that 37 mg/ml AGE could increase the production of IL-12. These observations suggest that AGE treatment can enhance IL-12 function, which is critically important for the control of intracellular *Leishmania* infection. The results of ELISA assays compiled from three experimental groups: intact macrophage without AGE, *Leishmania* infected macrophage without AGE, and *Leishmania* infected macrophage exposed with AGE.

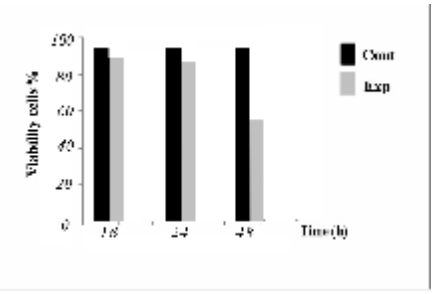

Fig. 1: MTT assay in macrophages on 18, 24 and 48 hours to compare with control group

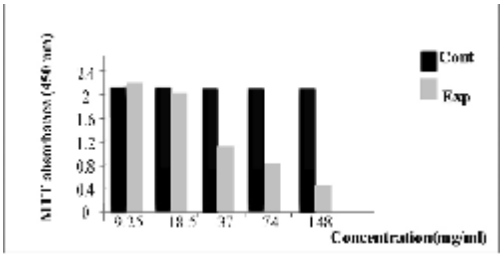

Fig. 2: MTT assay in macrophages on different concentrations AGE 9.25, 18.5,37, 74 and 148 mg/ml to compare with control group

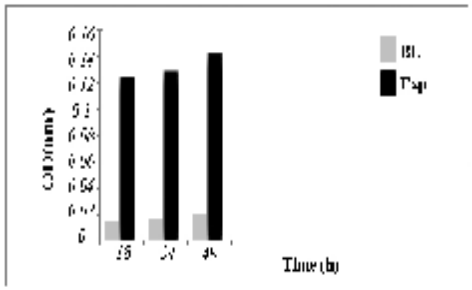

Fig. 3: IL-10 interaction in Leishmania infected macrophage without AGE treatment in 18, 24 and 48 hours to compare with control group

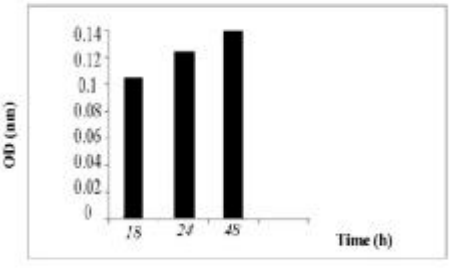

Fig. 4: Effects of AGE on Leishmania infected macrophage and interaction IL-10 in 18, 24 and 48 hours to compare with control group

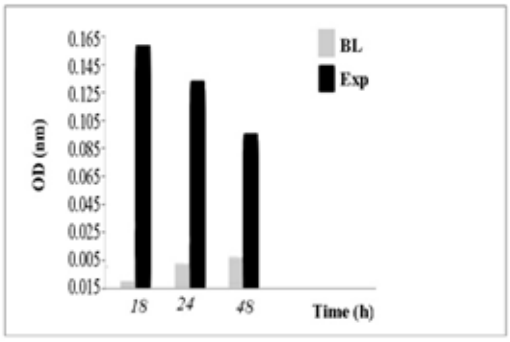

Fig. 5: IL-12 interaction in *Leishmania* infected macrophage without AGE treatment in 18, 24 and 48 hours to compare with control group

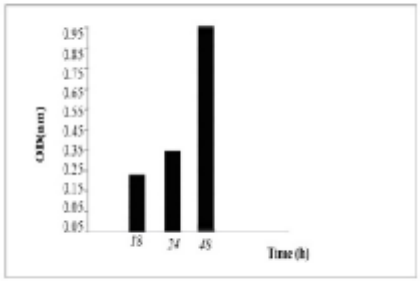

Fig. 6: Effects of AGE on *Leishmania* infected macrophage–induced IL-12 production in 18, 24 and 48 hours to compare with control group

Discussion

Some studies also investigated that when mouse macrophages were infected with amastigotes, they produced large amounts of IL-12. *Leishmania* parasites can also use LPG to inhibit the induction of IL-12. IL-12 is a major inducer of the Th1 cells, which produce IFN-γ, the substance that activates macrophages to produce NO.

The macrophage can produce IL-12 that following is IFN-γ then NO that Leishmanicide circuit is essentially complete. *Leishmania* parasites possess a number of survival mechanisms, one of, which is the switching off of the NO production machinery. Others also demonstrated that IL-12 was selectively expressed on Th1 cells but not on Th2 cells (2). Thus, we first determined the effective concentration of AGE required for induction *L. major* infected macrophage, because in vitro injection of high doses of AGE is toxic to the host (19). Thus, we exposed various doses 9.25, 18.5, 37, 74 and 148mg/ml of AGE during 18, 24 and 48 hours. In this report, we showed that 37 mg/ml of AGE (IC50: half maximal inhibitory concentration) in 48 hours into in vitro the *L. major* infected cell line is sufficient for IL-12 production by macrophage (2).

Treatment with AGE has several advantages. First, preparation of AGE is very easy. Second, we could change the dose of AGE to the minimum that is required for stimulation of macrophage. Third, AGE induce macrophage for IL-12 production and subsequent NO production, providing the best stimulation for induction of NO production. Fourth, may provide us with a highly effective means for the treatment of advanced leishmaniasis. Gurunathan et al. reported that substantiate the protective role of endogenous IL-12, we infected macrophage with the highly resistant background with *L. major*. Although they needed a longer period to achieve infection, they eventually healed, suggesting that endogenous IL-12 partially contribute to the host defense (20-21). In this study, we have shown that the *L. major* infected macrophage treated with various AGE concentrations induces IL-12 production but no IL-10. IL-10 can affect less for macrophage from progressive disease. Thus, IL-12 is essential for host defense, while IL-10 is not essential but may contribute to host defense mechanisms by hastening the period required for wound healing through the action to augment IFN-γ production. These and other studies suggested that IL-10 was not a key regulator in *Leishmania* infection, and that IL-10 did not play a role in T cell subset development. Recent

studies, however, have examined the role of IL-10 in IL-10-transgenic mice, in which the IL-10 gene was under the control of the MHC class II promoter (22). These mice had a profound phenotype and were highly susceptible to *L. major* infection. The susceptible phenotype of these transgenic mice indicates that the immunosuppressive activity of IL-10 on the macrophage/ monocyte population contributes to disease progression in leishmaniasis.

In conclusion, the study presented here has provided interesting preliminary data, which support the influence of roles for AGE in determining the outcome of *L. major* infection on macrophage. Our results show that dose of 37 mg/ml for 48 hours of garlic extract increased IL-12 secretion from infected macrophages. Therefore, IL-12 is crucial for defense against parasitic pathogens.

Ethical Considerations

Ethical issue principles including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc. have been completely observed by the authors.

Acknowledgements

This study was supported by grants No. 304 from Forensic Medicine of Iran University of Medical Science. We are grateful to Dr. Seyed Akbar Moosavi for critical reading of this manuscript and the Cellular and molecular Research Center and Biotechnology laboratory for their assistance. The authors declare that there is no conflict of interests.

References

1. Khalid NM, Mohomed HE, Toum AM, Mubark MA, Magzoub MA (2004). Treatment of Cutaneous leishmaniasis with some Local Sudanese Plants (Neem, Garad & Garlic). *Türkiye Parazitoloji Dergisi*, 28 (3): 129-132.
2. Ahmadi-Renani K, Mahmoodzadeh A, Cheraghali AM, Esfahani A (2002). Effect of garlic extract on cutaneous leishmaniasis and the role of nitric oxide. *IJMS*, 27(3): 97-100.
3. Mattner J, Wandersee-Steinhäuser A, Pahl A, Röllinghoff M, Majeau GR, Hochman PS, Bogdan C(2004). Protection against Progressive Leishmaniasis by IFN-β1. *J Immunol*, 172: 7574-82.
4. Khademvatan S, Gharavi MJ, Rahim F, Saki J (2011). Miltefosine induced apoptotic cell death on *Leishmania* major and *L. tropica* strains. *Korean J Parasitology*, 49(1):1-8.
5. Khademvatan S, Gharavi M J, Saki J (2011). Miltefosine induces metacaspase and PARP genes expression in *Leishmania infantum*. *Braz J Infect Dis*, 15(5): 442-48.
6. Anthony JP, Fyfe L, Smith H (2005). Plant active components - a resource for antiparasitic agents? *Trends Parasitol*, 21(10):462-8.
7. Ji J, Sun J, Soong L (2003). Impaired expression of inflammatory cytokines and chemokines a early stages of infection with *Leishmania amazonensis*. *Infect Immun*, 71:4278–88.
8. Jones DE, Ackermann MR, Wille U, Hunter CA, Scott A (2002). Early enhanced Th1 response after *Leishmania amazonensis* infection of C57BL/6 interleukin-10-deficient mice does not lead to resolution of infection. *Infect Immun*, 70:2151–58.
9. Kane MM, Mosser DM (2001). The role of IL-10 in promoting disease progression in leishmaniasis. *J Immunol*, 166:1141–47.
10. Murphy ML, Wille U, Villegas EN, Hunter CA, Farrell JP (2001). IL-10 mediates susceptibility to *Leishmania donovani* infection. *Eur J Immunol*, 31:2848–56.
11. Fiorentino DF, Bond MW, Mosmann TR (1989). Two types of mouse T helper

cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med*, 170:2081–95.

12. Moore KW, Malefyt RW, Coffman RL, Garra A (2001). Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol*, 19: 683–765.
13. Ralph P, Nakoinz I, Sampson-Johannes A, Fong S, Lowe D, Min HY, Lin M (1992). IL-10, T lymphocyte inhibitor of human blood cell production of IL-1 and tumor necrosis factor. *J Immunol*, 148:808–814.
14. Delaha EC, Garagusi VF (1985). Inhibition of mycobacteria by garlic extract (*Allium sativum*). *J Antimicrob Chemother*, 27: 485-88.
15. Panosian CB, Sypek JP, Wyler DJ (1984). Cell contact-mediated macrophage activation for antileishmanial defence. I. Lymphocyte effector mechanism that is contact dependent and noncytotoxic. *J Immunol*, 133: 3358-65.
16. Khademvatan S, Gharavi MJ, Akhlaghi L (2009). Induction of apoptosis by miltefosine in Iranian strain of *Leishmania infantum* promastigotes. *Iranian J Parasitol*, 4(2):23-30.
17. Khademvatan S, Saki J, Gharavi M J, Rahim F (2011). *Allium sativum extract* induces apoptosis in *Leishmania major* (MRHO/IR/75/ER) promastigotes. *J MED PLANTS* RES, 5(16): 3725–32.
18. Tsao SM, Hsu CC, Yin MC (2003). Garlic extract and two diallyl sulphides inhibit methicillin resistant *Staphylococcus aureus* infection in BALB/cA mice. *J Antimicrob Chemother*, 52: 974–980.
19. Jafari RA, Jalali MR, Ghorbanpoor M, Saraei SM (2008). Effect of dietary garlic on immune response of broiler chicks to live Newcastle Disease vaccine. *Pak J Biol Sci*,11(14):1848-51.
20. Gurunathan SD, Sacks L, Brown DR, Reiner SL, Charest H, Glaichenhaus N, Seder RA (1997). Vaccination with DNA encoding the immunodominant LACK parasite antigen confers protective immunity to mice infected with *Leishmania major*. *J Exp Med*, 186:1137–47.
21. Guy RA, Belosevic M (1993). Comparison of receptors required for entry of *Leishmania major* amastigotes into macrophages. *Infect Immun*, 61:1553-58.
22. Peyghan R, Powell MD, Zadkarami MR (2008). In vitro effect of garlic extract and metronidazole against *Neoparamoeba pemaquidensis*, and isolated amoebae from *Atlantic salmon. Pak J Biol Sci*, 11(1):41-7.