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Effect of *Tinospora cordifolia (Guduchi)* And LPS On Release Of H_2O_2, O_2^- And TNF-α From Murine Macrophages *In Vitro*

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Abstract

Macrophages are the first line of defense and constitute important participant in the bi-directional interaction between innate and specific immunity. Also macrophages are amenable to activation by biological response modifiers (BRM) of different origin. It has been found that certain BRMs impart their function with a distinct duality. They are capable of acting as immunopotentiators. *Rasayans* are immunomodulatory herbal drug preparations described in Ayurvedic system of medicine which exhibit a number of therapeutic properties, and it is thought that the mechanisms involved in these effects are due to the modulation of innate immunity and more specifically, macrophage function. This led us to investigate the effect of *Guduchi* (*Tinospora cordifolia*) and LPS a positive control on a macrophage cell line J774A. from J774A cells treated with *Guduchi,* and LPS showed enhanced production of hydrogen peroxide, superoxide and TNF-α levels. It is suggested that increased production of these products represent activated state of macrophages. These activated macrophages could kill the tumor cells via cytolysis mediated by the release of the secretary products like H_2O_2, O_2^- and TNF-α.

Key words: *Guduchi*, hydrogen peroxide (H_2O_2), LPS, macrophage activation, superoxide (O_2^-), TNF- α.

Introduction:

Macrophages have been recognized to play a crucial role in resistance to intracellular infection, [2,13,21] act as accessory cells in the immune response [8,11] and to function as cytotoxic cells acting against tumor cells [19] or virally infected cells [18]. Their ability to perform these various roles is related to their functional state. Resting macrophages when exposed to a variety of inflammatory agents undergo a range of changes. Such changes can lead to functional activities not expressed by the macrophage in its resting state and include alterations in enzyme content and secretion and enhanced phagocytic and bactericidal or tumoricidal effects.. Macrophages are quiescent cells which get activated when stimulated. Different types of agents such as antibiotics, antimetabolites and cytokines may exert an immunomodulating action that is expressed in the augmentation and/or inhibition of different immune responses [17]. One of the most promising recent alternatives to classical antibiotic treatment is the use of immunomodulators for enhancing host defense responses [25].

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A number of natural products and synthetic immunopotentiators termed as Biological Response Modifiers (BRMs) are becoming increasingly popular for testing their potential for augmenting immune responses. Among the natural BRMs many herbs and medicinal plants have long been known for their immunoaugmentary potential, however, only recently scientists have recognized them for their possible BRM actions. While our understanding of the mechanism of action of this BRM is still developing, it appears that the primary mechanisms involve induction of the immune response. The basic mechanism of the immunostimulatory, antitumor, bactericidal and other therapeutic effects of BRMs is thought to occur via macrophage stimulation. We have focused this study on the effect of BRMs on macrophage functions. We have investigated the effect of *Guduchi,* and LPS on the production of H_2O_2, O_2^- and TNF-α of macrophages. Macrophages play an important role in host defense by generating microbicidal oxygen metabolites including superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl ions. The respiratory burst is due to increased activation of NAD(P)H oxidase localized in the plasmalemma [6]. Analysis of the development of activation is facilitated when the operationally defined stages of activation is characterized using a library of markers for activation.

Materials and methods:

Reagents

RPMI 1640 with L-glutamine and 25 mM HEPES buffer was purchased from (HiMedia Pvt. Ltd. India.), Fetal bovine serum was purchased from Hyclone (Logan, USA) and heat inactivated at 56^0C for 45 min. Gentamicin (Ranbaxy Laboratories, Ltd. India) a sterile injectible antibiotic was commercially available in 80mg/2ml vials. The drugs (*Guduchi*) used were obtained from Himalaya Drug Company products, India. All other chemicals and solvents used in this study were obtained from Sigma Chemical Company (St. Louis, USA) and were of analytical grade or the highest grade available.

Cells

The macrophage J774A.1 cell line and the fibroblast L929 cell line was obtained from National Center for Cell Sciences (NCCS, Pune) used as source of macrophages, (Origin:BALB/c mouse; Nature:Mature) was grown and maintained in the Dulbecco's Modified Eagle Medium (DMEM) (pH 7.5) enriched with 10% fetal Bovine serum, at 37^0C and 5% CO_2 .

Viability assay

Cell viability was determined by the Trypan blue dye exclusion test. Equal volumes of cell suspensions were mixed with 0.4% Trypan blue in PBS, and the unstained viable cells were determined. These cells were further used for cytotoxicity assay in 2 x 10^6 densities per ml in the 96 well tissue culture plates.

Stimulation of macrophages

The macrophage cells (cell line J774A) from late log phase of growth (subconfluent) were seeded in 96 well flat bottom microtitter plates (Tarsons, India) in a volume of 100µl under adequate culture conditions. Drugs dissolved in medium were added in different concentrations in a volume of 100µl in triplicate. The cultures were incubated at 37^0 C and 5% CO_2 environment. After 24 hr and 48 hr incubation percent viability was checked and culture supernatants were collected and assayed for TNF- α activity. The inhibitory concentration (IC_{50}) of the drugs for the cells was estimated with MTT assay [26] and Trypan blue dye exclusion test. Rest of the assays was carried out with the minimum inhibitory drug concentrations.

H_2O_2 assay

H_2O_2 release by treated and untreated macrophage cultures (2x10^6 cells/ml) was quantified according to the method described elsewhere [16] with slight modifications. Phenol red was dissolved in HBSS (without phenol red) at a concentration of 0.1 g/l. Purified horseradish peroxidase (HRP) was dissolved in HBSS at a concentration of 165U/ml. Plates containing macrophages with the reaction mixture [containing 5 ml of phenol red solution, 0.16 ml of HRP and 500 ng/ml PMA (final concentration)] were incubated in CO_2 incubator for 1 h at 37^0C. At the end of incubation, 0.1 N NaOH was added and absorbance was measured at 615nm. For the standard curve, H_2O_2 (10- 100 µM) was prepared in quartz distilled water

O_2^- assay

Superoxide release was assayed by the method described by Johnston et.al. [10]. Briefly, 1.5 ml of reaction mixture containing 80 µM ferricytochrome C in HBSS without phenol red and PMA (500 ng/ml) was added to the 24h and 48h treated or untreated macrophage cultures (2x10^6 cells/ml).The cultures were then incubated for 90 min in a CO_2 incubator at 37^0C. After 90 min, the reaction mixture was transferred to chilled centrifuge tubes and centrifuged at 1000 rpm. for 10 min at 4^0C. The absorbance was measured at 550 nm and production of O_2^- calculated according to the formula:

Nanomoles of O_2^- released = 71.4 x OD_{550} .

A Bioassay for TNF activity

The activity of TNF-α in the culture supernatants of treated or untreated macrophages was assessed on L929 fibroblast cell line by a modification of the Mosmann method based on the reduction of MTT (Sigma) to a colored formazan by living cells [15]. Briefly, 2 x 10^{65}/well-L929 cells, in 100µl complete medium were grown in wells of a 96 well tissue culture plate in the presence of 1µg/ml of actinomycin D and 100µl of test culture supernatant. Cell viability was assessed after 24h of incubation. The supernatant was discarded and 10 µl of MTT;3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (3 mg/ml) was added to each well and plates were further incubated for 2h at 37°C. The enzyme reaction was then stopped by addition of 150µl of dimethyl sulfoxide (DMSO). Plates were incubated for 10 min under agitation at room temperature and colorimetric measurement of the MTT formazan was performed on an enzyme-linked immunosorbent assay plate reader at 570nm. Cells treated with culture supernatants of untreated macrophages were considered as Control. Percent viability and percent cytolysis of these cells was then calculated by the given formula.

$$\textbf{Percent viability} = \frac{E}{C} \times 100$$

Where, E is the absorbance of cells treated with culture supernatants of drug treated or untreated macrophages and C is the absorbance of cells treated with medium alone.

Percent cytolysis = (100 - percent viability).

Three independent experiments in triplicate were performed for the determination of TNF-α in the supernatants. TNF-α (mouse origin) in concentration of 10-100 pg was used as standard. The TNF- α levels were then calculated from the standard curve.

Statistical analysis

Statistical significance of difference between the control and experimental samples were calculated by Student's t-test. All the experiments were done in triplicate samples. Conclusions were drawn from 3 independent experiments.

Results:

H_2O_2 release

A significant increase in level of H_2O_2 (in nM/2x10^6 macrophages/ml) was observed in macrophages treated

with *guduchi* and LPS. And the results were compared with cells treated with medium alone. *Guduchi* treated macrophages showed an increase in H_2O_2 from 14.2 to 33. 91 nM for 24h and 48h of treatment respectively. Whereas, for macrophages treated with medium alone the yield of H_2O_2 remained low (14 to 18nM). ***(Table1)***

Table1: **Macrophages were treated with *guduchi*, LPS and medium alone for 24h and 48h and then checked for hydrogen peroxide release.**

Treatment		Hydrogen peroxide release (nM/ml)	
Drug	**Concentration (µg/ml)**	**24h**	**48h**
Guduchi	**80**	14.2±0.5	33.91±0.01*
LPS	**10**	15±0.5*	26±1*
Medium alone		14±0.05	18±0.05

The values are mean S.D. and representative of three different experiments done in triplicate. *P< 0.05; significantly different from respective controls.

O_2^- release

Macrophage J774A.1 cells ($2x10^6$) incubated in medium alone produced 12.54 and 13.7 nanomoles of superoxide (O_2^-) anions after 24h and 48h respectively. Cells treated with *guduchi* and LPS produced significantly higher amounts of O_2^- (16.6; 18.2 and 18.06; 18.4) as compared to controls after 24h and 48hours treatment respectively. It was observed that 48h treatment of drug for macrophages was most effective resulting in the release of O_2^-. After 48h treatment the drug treated cells showed increased O_2^- levels when compared to cells treated with medium alone.***(Table2).***

Table2: **Ferricytochrome C reduction (nanomoles/$2x10^6$cells/ml) in presence of PMA from drug treated and untreated macrophage cells (J774A.1)**

Treatment		Superoxide levels (nanomoles /ml)	
Drug	**Concentration (µg/ml)**	**24h**	**48h**
Guduchi	**80**	16.6±0.5	18.2±0.01*
LPS	**10**	18.06±0.5*	18.4±1*
Medium alone		12.54±0.05	13.7±0.01

TNF-α levels

Significantly elevated levels of TNF-α was found in the supernatants of macrophages treated with *guduchi* or LPS as compared to medium alone for both 24h and 48h treatment, respectively. ***(Table 3).***

Table3: **TNF-α levels in picogram/$2x10^6$ cells/ml from drug treated and untreated macrophage cells (J774A.1)**

Treatment		TNF- α levels pg/ml)	
Drug	**Concentration (µg/ml)**	**24h**	**48h**
Guduchi	**80**	80.46±0.5*	78.29±0.5*
LPS	**10**	71.26±1*	72.93±0.5*
Medium alone		24.70±0.5	17.61±0.25

Macrophages were treated with *guduchi*, LPS and medium alone for 24h and 48h and checked for TNF-α release. The values are mean S.D. and representative of three independent experiments done in triplicate. *P< 0.05; significantly different from respective controls.

Percent viability

The percent viability of L929 cells was checked after incubation with drugs treated or untreated macrophage supernatants. It was found that the cell viability of L929 cells was very high (100%) when treated with medium alone. While the cell viability of L929 cells drastically decreased when treated with macrophage supernatants (50%v/v) collected previously from macrophages treated with *guduchi* and LPS after 24h and 48h treatment, respectively. The percent viability of L929 cells on incubation with supernatants derived from *guduchi* treated macrophages was (25.14% and 29.63% ; 24h and 48h, respectively) significantly reduced as compared to viability of L929 cells treated with supernatants derived from macrophages treated with medium alone.(77.02% and 82.42%).

Whereas, the percent viability of L929 cells on incubation with supernatants derived from by LPS treated macrophages was (33.70% and 27.21%) after 24h and 48h treatment, respectively ***(Figure 1).***

Percent cytolysis

Percent cytolysis of L929 cells was calculated from percent viability as mentioned in Materials and Methods. It was found that the percent cytolysis of L929 cells was high (74.86% and 70.37%%; 24h and 48h, respectively) when incubated when incubated with *guduchi* and LPS (66.3% and 72.79; 24h and 48h, respectively) treated macrophage cell supernatants. On the other hand the percent cytolysis of L929 cells was found to be low (22.98 and 17.58; 24h and 48h, respectively) with macrophage supernatants treated with medium alone. Percent cytolysis of L929 cells by drug treated macrophage supernatants was significantly different than with the % cytolysis by macrophage cell supernatants treated with medium alone. No cytolysis was observed in cells treated with medium alone ***(Figure 2).***

***Fig 1 and 2*: Percent viability and percent cytolysis of L929 cells respectively:**

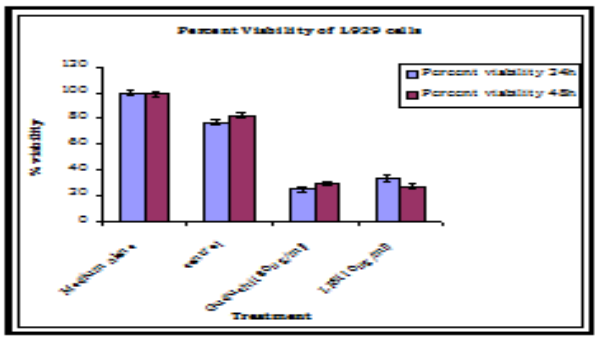

***Fig1*:** Macrophages were treated with *guduchi*, LPS alone for 24h and 48h and then checked for superoxide release. The values are mean S.D. and representative of three different experiments done in triplicate. *P< 0.05; significantly different from respective controls.

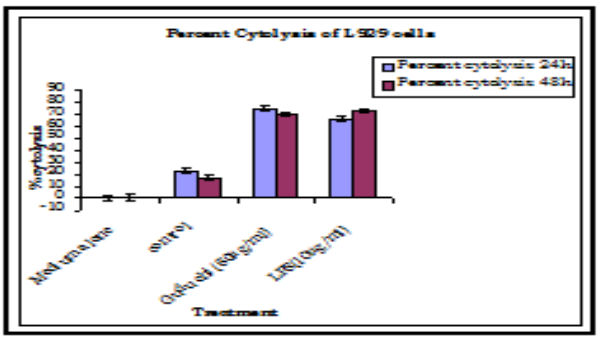

***Fig2*:** Macrophages were treated with *guduchi*, LPS alone for 24h and 48h and then checked for superoxide release. The values are mean S.D. and representative of three different experiments done in triplicate. *P< 0.05; significantly different from respective controls.

Discussion:

Macrophages are considered to play a central role in host's antitumor immune responses. Macrophages can recognize and eliminate tumor cells. To this effect these cells use a variety of cytotoxic effectors. Nitric oxide and its metabolic byproducts mediate macrophage tumor cytotoxicity. Cytotoxic reaction is the ultimate indicator of activated macrophages. These cyotoxic effects include various proteases, reactive oxygen intermediates, TNF-α and reactive nitrogen species such as nitric oxide [1].Our findings show that *guduchi* and LPS treated macrophages significantly enhanced the hydrogen peroxide (H_2O_2), superoxide (O_2^-) and TNF-α level ***(Table 1, 2 and 3)*** as compared to macrophages treated with medium alone. It also showed enhanced macrophage mediated cytolysis ***(Figure 1 and 2)*** against L929 cells. The L929 fibroblast cells were treated with supernatants derived from treated or untreated macrophages to check cytolysis. It was found that the percent viability of L929 cells was low when treated with supernatants derived from *guduchi* or LPS treated macrophages, whereas an increase in percent cytolysis was observed after 24h and 48h treatment. ***(Table3)***. Macrophages are dynamic cells which are the first line of defense. Macrophages perform a variety of complex microbicidal functions, including surveillance, chemotaxis, phagocytosis and destruction of targeted organisms. The spectrum of microorganisms are kept on check by phagocytes includes fungi, bacteria and virus infected cells [4]. Recent evidences suggest that development of preventative strategies to resist disease could certainly be a more efficient and possibly more effective long-term healthcare strategy [9,12]. The development of novel therapeutics, which could nonspecifically augment the innate immune response, represents an ideal strategy for addressing current worldwide concerns of how to combat classical and emerging infectious agents. One of the most promising recent alternatives to classical antibiotic treatment is the use of immunomodulators for enhancing host defence responses[25]. Plant derived immunomodulatory compounds have also been used in traditional remedies for various medical problems and the investigation of these sources has grown exponentially in recent years. While our understanding of mechanism of action of these substances is still developing, it appears that one of the primary mechanisms involves nonspecific induction of the immune system [25].India has a rich tradition in the treatment of many diseases by therapy with '*Rasayans*.' In Ayurveda '*Rasayans*' are concerned with nourishing body and boosting immunity. They are also modulators of the immune system and one such cell modulated by them is (***Figure 2***). As mentioned in the Materials and Methods, macrophages were initially treated with the *guduchi* and LPS and then assessed for hydrogen peroxide and superoxide release. Macrophages also showed high levels of hydrogen peroxide (H_2O_2) and superoxide (O_2^-) when treated with *guduchi*(80µg/ml) or LPS(10µg/ml) for 24h and 48 h. Also *guduchi* (80µg/ml) treatment led to enhanced TNF- α production by macrophages.

The alcoholic and aqueous extracts of *guduchi* have been tested successfully for immunomodulatory properties [2,14,20,23]. It has been also observed that extracts of several medicinal plants stimulate the macrophages [3,5,24]. Findings of earlier studies suggested that most of the therapeutic compounds in plants are polar and require polar solvents for their extraction. However, the ethanolic extract failed to show significant activity in the present study. The herbs are being consumed by people since long as medicines. The results of present study show experimental basis of immunomodulation by biological response modifiers (BRMs).

It is obvious that man, despite developing many new technologies to understand the host pathogen interrelationship is still far from conquering the microbes. Pathogens are adapting to challenges put forth by man in the battle against it by developing resistance to the multitude of drugs currently available. There is thus a great need to understand how exactly pathogen survives in vivo conditions and identify pathways unique to the intracellular environment that could be utilized for the development of new and better drugs. Today it is becoming clearer that the products identified under the traditional or alternative medicines in vitro conditions also stimulate the natural in vivo conditions and thus may give the true direction to the problem. Studies are underway to check the effect of these preparations on activation of human peripheral blood monocytes.

Conclusion:

This study addresses a very pertinent question of bio-medical sciences dealing with the scientific basis particularly related to immuno-modulatory effects of the herbal medicine preparation on the macrophage activation as macrophages are known to represent the first line of defense against invading microorganisms or in a state of altered self.

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References:

1. Adams, DO., and Nathan, C.F. Molecular mechanisms in tumor-cell killing by Activated macrophages. Immunol. Today 4.166. 1983.

2. Alan Aderem and David M. Underhill (1999) Mechanism of phagocytosis in macrophages Annu. Rev. Immunol. 17:593–623.

3. Atal et al., 1986. C.K. Atal, M.L. Sharma, A. Kaul and A. Khajuria,Immunomodulating agents of plant origin. I. Preliminary screening. *Journal of Ethanopharmacology* 41 (1986), pp. 185–192.

4.Beutler B (2004). Innate immunity: an overview. Molecular Immunology 40: 845-859.

5.Broker R, Bhatt JV (1953). Symposium on anti-bacterial substances from soil, plants and other sources. XV. Phagocyticcoefficient as a measure for evaluating plant antibiotics. Ind J Pharm. 309-10.

6.Dewald B, Baggiolini M, Curnutte I J and Babior B M, J Clin. Invest, 63(1979)21.

7.Dikshit V, Damre AS, Kulkarni KR, Gokhale A, Saraf MN (2000). Preliminery screening of immunocin for immunomodulatory activity. Ind J Pharm Sci.62, 257.

8.H Furuie, H Yamasaki, M Suga, M Ando, Altered accessory cell function of alveolar macrophages: a possible mechanism for induction of Th2 secretory profile inidiopathic pulmonary fibrosis Eur Respir J 1997; 10: 787–794.

9.Hackett CJ. Innate immune activation as a broad spectrum biodefense strategy: prospects and research challenges. J Allergy Clin Immunol 2003; 112:686-94.

10.Johnston RB Jr, Chadwick DA, Cohn ZA. Priming of macrophages for enhanced oxidative metabolism by exposure to proteolytic enzymes. J Exp Med 1981;153:1678-83.

11.K Miller, B N Hudspith, and C Meredith Secretory and accessory cell functions of the alveolar macrophage.Environ Health Perspect. 1992 July; 97: 85–89.

12.Lolis E.Bucala R. Therapeutic approaches to innate immunity; severe sepsis and septic shock. Nat Rev Drug Discov 2003;2:635-45.

13.Mackaness, G.B. 1964. The Immunological basis of acquired cellular resistance. J.Exp. Med. 120:105–120.

14.Manjrekar PN, Jolly CI, Narayanan S(2000). Comparative studies of the immunomodulatory activity of *Tinospora cordifolia* and *Tinospora sinensis*. Fitoterapia.71, 254-7.

15.Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival:Application to proliferation and cytotoxicity assays. J. Immunol. Methods 65: 55-63.

16.Pai K, Sodhi A. Effect of cisplatin, r-IFN-γ, LPS and MDP on release of H_2O_2, O_2^- and lysozyme from human monocytes *in vitro*. Ind J Exp Biol 1991; 29:910-5.

17.Pai, K., Shrivastava, A., Kumar, R., Khetarpal, S., Sarmah, B., Gupta, P. and Sodhi,A. (1997). Activation of P388D1 macrophage cell line by chemotherapeutic drugs.Life Sciences, 60(13):1239-48.

18.Paul A. LeBlanc (1989) Macrophage activation for cytolysis of virally infected target cells. Journal of Leukocyte Biology 45:345-352.

19.Pinedo HM, Slamon DJ (2000) Translational research: the role of VEGF in tumor angiogenesis. Oncologist 5: Suppl 11–2 (Article).

20. Rege NN, Dahanukar SA, Thatte UM, (1999). Adaptogenic properties of six *Rasayana* herbs used in Ayurvedic medicine . Phytother Res . 13, 275-91.

21.Singh, N., Singh, S.M., Shrivastava, P. (2005). Effect of *Tinospora cordifolia* on the antitumor activity of tumor associated macrophages-derived dendritic cells. Immunopharmacology and Immunotoxicology, 27(1): 1-14

22.Singh, S.M., Singh N. and Shrivastava, P. (2006). Effect of alcoholic extract of Ayurvedic herb *Tinospora cordifolia* on the proliferation and myeloid differentiation of bone marrow precursor cells in a tumor bearing host. Fitoterapia,77:1-11.

23.Thatte UM, Dahanukar SA (1989). Immunotherapeutic modification of experimental infections by Indian medicinal plants. Phytother Res. 3, 43-9.

-

24.Thatte UM, Rao SG, Dahanukar SA (1994). *Tinospora cordifolia* induces colony stimulating activity in serum. J. Postgrad Med. 40, 202-3.
25.Tzianabos AO. Polysaccharide immunomodulators as a therapeutic agents: structural aspects and biological function. Clin Microbiol Rev 2000; 13: 523-33.
26.More PM, Pai K. Immunomodulatory effects of *Tinospora cordifolia* (*Guduchi*) on Macrophage activation. Biology and Medicine 2010 (Accepted).