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ACTIVATION OF MURINE MACROPHAGES BY FROG PEPTIDE ISOLATED FROM SKIN OF *RANA TIGRINA*

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ABSTRACT
A number of different bio-reactive molecules such as peptides, steroids, alkaloids and opiods have been isolated from amphibian's skin that possess potent anti-bacterial, anti-fungal, anti-protozoan, anti-cancer and anti-diabetic effects. Amphibian skin derived-peptide can provide potential dimension towards the development of newer drugs to combat several pathological conditions. The aim of this study was to investigate the effect of frog peptide isolated from the skin of *Rana tigrina* on the activation of macrophages. Production of reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) were measured with the help of ELISA. Our findings revealed that *in vitro* treatment of macrophages with frog peptides resulted in significant increase in the production of RNI and ROI as compared to untreated or control group of macrophages. The maximal production of RNI and ROI was observed only after 24 hrs of incubation in complete culture medium at 100µg/ml peptide concentration and this effect was dose dependent at lower concentrations. This indicates that frog peptide obtained from the skin of *Rana tigrina* is capable of activating murine macrophages for the enhanced production of non-specific effector molecules (RNI and ROI) that may play a significant role in macrophage-mediated immunity.

KEY WORDS: Frog peptide, Dalton's lymphoma, macrophages, *Rana tigrina*, RNI, ROI.

INTRODUCTION

The macrophage plays an important role in innate and adaptive immune responses. Macrophages have been considered as the first line of defense mechanism against infections and malignancies in the host (Morimoto and Santaro, 1998; Kaufmann, 1990). Upon activation, the macrophages produce various specific and non-specific effector molecules including pro-inflammatory cytokines and chemokines necessary for recruitment of lymphocytes at the site of antigen entry or tumor progression and show profound bactericidal and tumoricidal activity (Mosser, 2003). It has been found that tumor cells are able to suppress the function of macrophages and skews it to M2 phenotype or alternatively activated macrophage population in the tumor-bearing host. Therefore, in the tumor-bearing host, the cytotoxic functions of macrophages get altered, which invariably results into tumor development and progression. Skin secretions from frogs belonging to the family Ranidae (''true frogs'') have proved to be a particularly rich source of antimicrobial peptides and more than 200 such peptides from approximately 60 species have been described (Conlon *et al.*, 2004 and 2009). At least four such peptides (Tigerinin 1, Tigerinin 2, Tigerinin 3, Tigerinin 4) have been isolated from the skin of Indian bull frog *Rana tigrina*, which is the most abundant and easily available species of frog in Indian continent (Sai *et. al.*, 2001). Therefore it could be a cheap source of bioactive peptides.

The important goal of this study was to determine whether skin peptides from *Rana tigrina* could activate macrophages in tumor bearing mice to inhibit growth of tumor progression. In the present study, the active peptide isolated from the dorsal skin of *Rana tigrina* has been used for activation of normal and tumor associated (Dalton lymphoma) macrophages of experimental mice. Reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) assays have been performed to evaluate the activity of skin peptide. After treatment with frog peptide significant increase in the level of RNS (Reactive nitrogen species) and ROS (Reactive oxygen species) production have been observed in both normal as well as tumor associated macrophages (TAM) of experimental mice, which indicates that the peptide has the potential to be used as drug for the treatment of tumors and infections as these effector molecules (RNI and ROI) play crucial roles in cell mediated immunity through inflammatory mechanism.

MATERIALS AND METHODS

Inbred strains of healthy BALB/c ($H2^d$) mice of either sex at 8 – 10 weeks of age were used for the experiments. The mice were maintained in utmost hygienic conditions with clean water supply and proper lighting. The mice were killed by painless method, cervical dislocation for the isolation of peritoneal macrophages. For tumor model, a cell line of T-cell lymphoma of spontaneous origin – Dalton lymphoma (DL) was selected. The mice of either sex were injected intra peritoneally with 1.5 X 10^6 DL-cells in 0.5 ml of PBS. The DL-cells were maintained *in vivo* by serial transplantation or cryopreserved for future use. The DL-cells isolated from the DL-bearing host were used for transplantation only at the day when the yield of DL-cells was higher.

Frog peptides were isolated by a standard method described by Amanda *et. al.* (2001) and protein content was estimated by standard Lowry method (Oliver *et al.*, 1951).

Macrophage isolation

Mice with and without tumor were killed by cervical dislocation and peritoneal exudates cells (PECs) were harvested by peritoneal lavage using chilled serum free media RPMI 1640 as described by Sodhi *et. al.*,1992. PECs were then cultured in tissue culture flask at 37 ^{o}C in a CO_2 incubator for 2 hours. The cultures were then washed three times (3X) with warm serum free media with gentle flushing to ensure that all the DL or other non-adherent cells were removed; 95% of the adherent cell population were macrophages as determined by morphology and non-specific esterase staining. These tumor associated macrophages and normal macrophages were detached from tissue culture flask with a cell scrapper and plated in 96 well flat bottom culture plate. Nitrite (NO_2) a product of nitric oxide (NO) was used as an indicator for nitric oxide. Nitrite concentration in culture supernatant was determined by a microplate assay described by Ding *et. al.*, 1988. H_2O_2 release was measured using the horse radish peroxidase dependent phenol red oxidation micro assay (Pai and Sodhi, 1991). Two tailed Student t-test and Sigma Plot version 12.0 were used for data analysis and presentation. The differences in the results were considered significant at $p<0.05$

RESULTS

Reactive nitrogen intermediate (RNI) assay

For RNI assay 1.5 X 10^5 macrophages were incubated with medium only or medium containing LPS, peptides or LPS + peptides for 24 hours and nitric oxide production was measured as nitrite release in the culture supernatants. The data are representative of three independent experiments done in triplicate, and are represented as mean concentration of nitrite ± SE_M. The symbol * indicates the significant increase in nitrite production after peptide treatment than the medium only or LPS treated macrophages, ** indicates significantly higher nitrite production in macrophages treated with peptides + LPS than the macrophages treated with peptides alone, and # indicates the higher nitrite release by TAMs treated with peptides than the normal treated or untreated macrophages. We have here measured nitrite production on proper stimulation of peritoneal macrophages from both normal and DL-bearing mice. The result has been compared with double control; untreated (negative) and LPS treated (positive) as a standard macrophage activating agent for macrophage activation. As the result shows, there is significant increase in the production of nitric oxide (NO) as compared to untreated and LPS treated macrophages. The result indicates that the peptides significantly enhance the production of NO_2 in treated macrophages as compared with normal macrophages treated with only control. LPS has been reported as a potent macrophage activating agent, but frog peptide is also a good activating agent in comparison to LPS. In combination with LPS the peptide further enhances the production of NO significantly (Figure 1). This experiment has been done in DL-bearing mice. The tumor associated macrophages (TAMs) also show the enhanced production of nitric oxide following treatment with peptides indicating that peptides are able to revert back the immunosuppressed phenotype, designated as M2 phenotype of macrophages, into a more tumoricidal or M1 phenotypes, indicating tumoricidal potential of the frog peptide isolated from *Rana tigrina*.

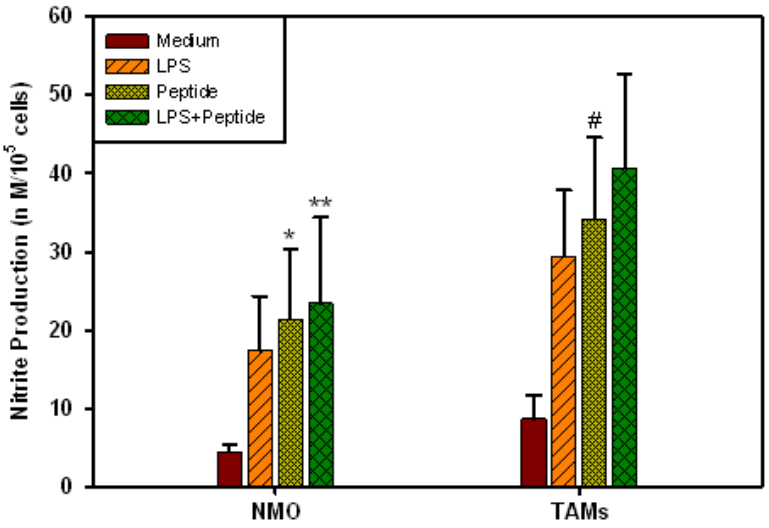

Figure 1. Effect of the skin peptides on the nitrite production.

Reactive oxygen intermediate (ROI) assay

For ROI assay also 1.5 X 10^5 macrophages were incubated with medium only or medium containing LPS, peptides or LPS + peptides for 24 hours and Hydrogen peroxide (H_2O_2) production was measured in the culture supernatants. The data are representative of three independent experiments done in triplicate and are represented as mean concentration of H_2O_2 in the culture supernatants ± SE_M. The symbol * indicates the significant increase in H_2O_2 production in peptide treated macrophages than the medium or LPS treated macrophages, ** indicates significantly higher production of H_2O_2 in peptide + LPS treated macrophages than the macrophage treated with peptide or LPS alone, and # indicates the higher H_2O_2 release from TAMs treated with peptides than normal macrophages treated or untreated.

Statistically significant increase in the production of H_2O_2 was observed in normal resident macrophages and TAMs after *in vitro* stimulation by peptides. The result is shown in Fig. 2. On comparison between normal and TAMs treated with LPS, peptides, LPS + peptides, it has been observed that TAMs show significantly higher production of H_2O_2 in comparison to control and LPS treated macrophages, and in combination with LPS and peptides shows little bit higher production of H_2O_2. This result also shows that peptide has an augmentary effect on activation of macrophages for the production of ROI.

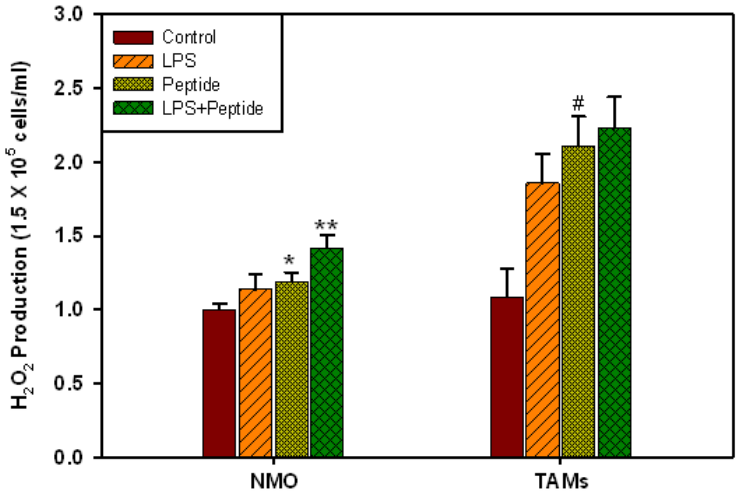

Figure 2. Effect of skin peptide on H_2O_2 production.

DISCUSSION AND CONCLUSION

The findings of present research suggest that the frog peptides isolated from *Rana tigrina* directly or indirectly induce the production of reactive intermediates metabolites in normal and altered macrophages (TAMs). *In vivo* treatment with the peptides showed enhanced production of NO and H_2O_2, by TAMs. Macrophages are considered as the first line of defense in conferring innate immunity against infection and malignancies. However, tumor growth simultaneously modulates their functions (Ohashi *et. al.,* 2000), and as a result they lose their normal functions and eventually have tumor protective function. Usually, TAMs produce several cytokines of immunosuppressive function or tumor growth promoting function, such as IL-10 and TGF-β and other suppressive mediators such as prostaglandin. In the present study higher production of nitric oxide and hydrogen peroxide was observed by TAMs after treatment with frog peptides in DL-bearing mice through the enzymatic action of iNOS (inducible Nitrogen oxide synthase) on terminal guanidinonitrogen of L-arginine that yield L-citrulline as co-product. Therefore, the release of these NO_2, H_2O_2 and TNF-α are considered as major tumoricidal action of activated macrophages *in vitro* (Hibbs *et. al.,* 1988; Stuehr *et. al.,* 1989; Cui *et. al.,* 1994) and *in vivo* (Yim *et. al.,* 1993; Farias-Eisner *et. al.,* 1994).

TAMs have different characteristics than their normal counterpart due to association with tumor. Since, results confirmed the enhanced activity of TAMs upon activation by frog peptides; it can be assumed that this increased activity of TAMs is due to reversal of their altered function towards normal which could be relevant in the formulation of immunotherapeutic protocols against malignancies. Normally, most of the BRMs (Biological Response Modifiers) that are constitutively synthesized and localized in the cytosol; their expressions are induced in inflammatory conditions (Soltys *et al.,* 1996 and 1997; Laplante *et al.,* 1998) such as in malignancies and other infectious diseases. Necrosis of these inflamed cells results in the higher local concentration of certain factors including Hsp70 in extra cellular microenvironment which stimulates macrophages and other immune cells and triggers the immunologically relevant genes. Un-stimulated macrophages do not show any changes at synthesis level as well as in the production of reactive intermediates. Therefore, it can be concluded that peptide treatment must exert stimulatory effect at the level of transcription and expression of the immunologically relevant genes. This study demonstrates anticancer activity of skin

peptides isolated from Indian bull frog *Rana tigrina*. We report the immunomodulatory potential of frog peptide with an exceptionally large spectrum activity against Dalton's lymphoma. In this study, we have shown that the peptides derived from the skin of *Rana tigrina* are capable to restore not only the suppressed function of TAMs, but also enhance the production of various effector molecules and proinflammatory molecules.

REFERENCES

Amanda L. Salmon., Laurence J. M. Cross., Alexadra E. Irvine., *et. al.* (2001). Peptide Leucine Arginine, A potent immunomodulatory peptide isolated and structurally characterized from the skin of the Northern leopard frog, *Rana pipiens. JBC.* 276 (13): 10145-10152.

Cui S., Reichner J., Mates R. and Albina J. (1994). Activated murine macrophages induces apoptosis in tumor cells through nitric-oxide dependent or independent mechanism. *Cancer Res*. 54: 2462-67.

Ding A. H., Nathan C. F. and Stuehr D. J. (1988). Release of reactive nitrogen intermediate and reactive oxygen intermediate from mouse peritoneal macrophages, *J. Immunol.* 149: 3290-96.

Farias-Eisner R., Sherman M., Aeberhard E. and Chaudhuri G. (1994). Nitric oxide is an important mediator of tumoricidal activity *in vivo*. *Proc. Natl. Acad. Sci. USA*. 91: 9407-9411.

Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun*. 157: 87-98.

Laplante A. F., Moulin V., Auger F. A., Landry J., Lie H., Morrow G., Tanguay R. M. and Germain L. (1998). Expression of heat shock proteins in mouse skin during wound healing. *J. Histochem. Cytochem*. 46: 1291-98.

Ohashi K., Burkart V., Flohe S. and Kolb H. (2000). Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *J. Immunol*. 164: 558-61.

Oliver H. Lowry., Nira J., Rosebrough A., Lewis Farr and Rose J. Randall (1951). Protein measurement with the Folin Phenol Reagent. *J. Biol. Chem.* 265-275.

Pai K. and Sodhi A. (1991). Effect of cisplatin, rIFN-γ, LPS and MDP on release of H_2O_2, O_2 and lysozyme from human monocytes *in vitro*. *Ind. J. Exp. Biol*. 29: 910-15.

Sodhi A., Singh R. K. and Singh S. M. (1992). Effect of interferon gamma priming on the activation of murine peritoneal macrophages to tumoricidal state by cisplatin, interleukin-1 and TNF. *Clin. Exp. Immunol*. 88: 350-355.

Soltys B. J. and Gupta R. S. (1996). Immunoelectron microscopic localization of the 60kDa heat shock protein (hsp60) in mammalian cells. *Exp. Cell Res*. 222: 16-23.

Soltys B. J. and Gupta R. S. (1997). Cell surface localisation of the 60 kDa heat shock chaperonin protein (Hsp60) in mammalian cells. *Cell. Biol. Int.* 21: 315-21.

Stuehr D. and Nathan C. (1989). Nitric Oxide: a macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *J. Exp. Med*. 169: 1543-50.

Yim C., Bastian N., Smith J. C., Hibbs J. J. and Samlowski W. (1993). Macrophage nitric oxide synthesis delays expression of ultraviolet light induced murine skin cancer. *Cancer Res*. 15: 5507-14.

