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Research Article

# NITRIC OXIDE SYNTHESIS FROM ACTIVATED MONOCYTES INDUCED BY FRACTIONATED SOLUBLE (CYTOPLASMIC) ANTIGEN OF *B. MELITENSIS* 16M IN CELL CULTURE.

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## ABSTRACT

**The present research was designed to investigate the production of nitric oxide by monocytes in cell culture induced by different fractionated soluble antigen of *Brucella melitensis* 16M. For this purpose soluble antigen of *B. melitensis* was prepared by treating the culture with ultrasonic waves in an ultrasonicator. This antigen was then subjected to gel filtration chromatography to fractionate the antigen according to its approximate molecular weight. Nitrite and other reactive intermediates have been postulated to participate in arginine dependent tumor cytotoxicity by activated macrophages, supernatant were collected after 48 hr. of culture assayed for the presence of these molecules in a standard Griess reaction. This investigation revealed that fractionated antigen SAG3 produced maximum nitrite followed by SAG1, SAG2, SAG5, SAG4 and SAG6 respectively. Therefore, it was observed that NO play an important role in killing of *B. melitensis* and it had been concluded that much of the antimicrobial activity of macrophages against *B. melitensis* is due to nitric oxide and substances derived from it.**

**Key words: *B. melitensis*, Nitric oxide, macrophages, Griess reaction, soluble antigens.**

## INTRODUCTION

Nitric oxide has potent antimicrobial activity; it can also combine with the superoxide anion to yield even more potent antimicrobial substances. Recent evidence suggests that much of the antimicrobial activity of macrophages against bacterial, fungal, helminthic and protozoal pathogens is due to nitric oxide and substances derived from it.

Brucella is a pathogenic bacterium that has been called "exceedingly well adapted" to the niche of living in compartments within professional and

nonprofessional phagocytes (Moreno and Moriyon, 2002). It is found all over the world infecting many different types of mammals from marine mammals to horses to humans. Brucella can survive inside macrophages and are facultative intracellular pathogens; they enter their host through mucosal surfaces. The bacterium is able to do this because it is gram-negative and can present itself upon culture with a smooth or rough colony morphology because it is possible for smooth colonies to spontaneously become rough and some rough colonies to spontaneously become smooth (Schurig *et al.*,1981).

Brucella have opsonized with antiserum to ensure the infection of macrophages during in vitro studies (Elzer *et al.,* 1994). Both non-opsonized and antibody opsonized *B. suis* strains induce m RNA for inducible nitric oxide (NO) synthase (iNOS) in the mouse macrophage like J 774A.1 cell line. However, only antibody opsonized *B. suis* triggers NO production. Gamma interferon (IFN-γ) induced NO mediated bacteriostasis occurs preferentially if Brucella are opsonized with antibody (Gross *et al.,* 1988). The contribution of opsonins to the fate of ingested *B. melitensis*, however, has not been systematically examined. In addition the inter play of opsonins in the clearance of Brucella from primary macrophages, rather than the cell line, is unknown.

Inside macrophages, Brucella survives by inhibiting the phagosome-lysosome fusion. Within placental trophoblasts and other non-professional phagocytes, *Brucella* has been shown to localize and proliferate in autophagosome-like compartments resembling the rough endoplasmic reticulum (Moreno and Moriyon, 2002). Similarly, *B. abortus* localizes and replicates in the rough endoplasmic reticulum of trophoblastic epithelial cells in pregnant ruminants (Detilleux *et al*., 1990). However, most of the mechanisms that allow the bacteria to cause tissue tropism as well as the way in which Brucella enters and lives in such different host cells such as epithelial cells are not known.

## MATERIALS AND METHODS

*B. melitensis* sonicated supernatants were fractionated by gel filtration on a 1.5 X 80 cm column bed of sephacryl S-200 having a total volume ($V_t$) of 136 ml. Void volume ($V_0$) of the column bed was 45 ml. The column was pre-calibrated with standard protein molecular weight marker viz. bovine serum albumin (MW 66 KDa) and cytochrome C (MW 12.5 KDa). 2 ml of concentrated sonicated antigen containing 80 mg total protein was eluted at the rate of 16 ml per hour with 30 mM Tris buffer (pH 7.5) containing 0.1 M sodium chloride. Absorbance of elutes was monitored at 280 nm. Fractions thus obtained in each region were pooled and were dialyzed against distilled water at $4^oC$. All the pooled fractions were concentrated by vaccum concentrator and filter sterilized through a membrane filter (0.22 µm), aliquoted and stored at $-20^oC$.

Nitrite and other reactive intermediates have been postulated to participate in arginine dependent tumor cytotoxicity by activated macrophages, supernatant were collected after 48 hr. of culture assayed for

the presence of these molecules in a standard Griess reaction. Nitric oxide test was performed as per the method of Stephanie *et al.,* (1989). For this test 5 experimental goats, which were previously immunized with the soluble antigens and one unimmunized goat was used as control. Blood was collected separately from external jugular vein of these goats into sterilized assembly containing 2.7% EDTA in ratio of 20:1. Peripheral blood mononuclear cells (PBMNCs) were isolated from 10 ml of venous blood by centrifugation at 2000 rpm on histopaque at room temperature for 30 min. PBMNCs were removed from the interphase and were washed three times in sterilized PBS by centrifugation at 1000 rpm for 10 min. After washing PBMNCs were suspended in growth medium [DMEM supplemented with 10% FBS, 2 mM L-glutamine, penicillin-streptomycin (100U/ml), gentamicin (50μg/ml) and 2- mercaptaethanol] were cultured at 2X $10^5$ cells/well in 96 wells round bottom microtiter plates in triplicate wells, three wells without antigen were used as controls. Plates were incubated at $37^0$C under 5% $CO_2$ for 48 hours. After the completion of 48 hours incubation 50 μl of supernatant was harvested and added in a new 96 well microtiter plate by using multichannel, this supernatant was used in NO test. Nitrite concentration in the supernatant was assayed by a standard Griess reaction. 50 μl of supernatant were added to 100 μl of 1% sulfanilamide, and then 100 μl of 0.1% naphthylethylene diaminedihydrochloride was added. Plate was incubated at $37^0$C for 20 min. The absorbance was read at 543 nm in ELISA reader.

**RESULTS AND DISCUSSION**

The approximate molecular weight of each fraction collected for soluble antigens was identified by gel filtration chromatography (Table 1). When we used fractionated soluble antigen for the induction of nitric oxide, it was observed region SAG3 produce maximum nitrite followed by SAG1, SAG2, SAG5, SAG4 and SAG6 respectively (Table 2).

For several intracellular pathogens, a lack of correlation in the effects of varying NO levels on microbial burden has led to different conclusions regarding the role of NO as an anti-pathogen effector. For example murine macrophage NO may act alone or cooperate with other macrophage microbicidal mechanisms to kill Candida (Vazquez *et al*., 1995). In case of *L. monocytogenes*, murine macrophage NO may play a direct listericidal role in primary infections but may be of less importance in secondary infections (Samson *et al*., 1996). NO is involved in the elimination of *B. suis* from murine cells, provided that both Brucella antibodies and IFN-γ are present. Non-opsonized bacteria did not trigger production of iNOs or NO, although they did not enhance iNOs m-RNA levels (Jiang *et al*., 1993 and Gross *et al*., 1998). In this study also all the fractionated soluble antigen induces the production of nitric oxide, but fractionated antigen SAG3 induces more nitrite production followed by SAG1, SAG2, SAG5, SAG4 and SAG6 respectively. We have also observed experimentally that the lymphocyte cultured from the goat which were previously

immunized with soluble antigen and again induced by fractionated soluble antigen in cell culture able to induce the synthesis of NO *in vitro*. Therefore, we also agree that NO play an important role in killing of *B. melitensis* and our study agrees with the previous studies.

**Table 1. Table showing peaks, regions and molecular weight range of soluble antigens.**

| Soluble Antigens (SA) | | |
|---|---|---|
| **Peak** | **Region** | **Mol. Wt Range (KDa)** |
| I | SAG1 | 0 – 80 |
| II | SAG2 | 84 – 78 |
| III | SAG3 | 78 – 66 |
| IV | SAG4 | 66 – 45 |
| V | SAG5 | 45 – 23.5 |
| VI | SAG6 | 23.5 – 12.5 |

SAG: Soluble antigen fractioned by Gel filtration chromatography

**Table 2. Nitrite production from activated monocytes to various *B. melitensis* soluble antigens**

| **Peaks** | **Nitric Oxide Concentration (µM)** | | | | | |
|---|---|---|---|---|---|---|
| | **Animals** | | | | | |
| | **S-1** | **S-2** | **S-3** | **S-4** | **S-5** | **C** |
| **SAG1** | 18.32 | 19.71 | 16.30 | 19.01 | 17.01 | 0.97 |
| **SAG2** | 16.02 | 17.03 | 18.03 | 17.75 | 19.56 | 0.87 |
| **SAG3** | 19.02 | 20.13 | 21.71 | 22.01 | 20.03 | 0.99 |
| **SAG4** | 12.3 | 14.13 | 16.01 | 15.03 | 19.04 | 0.88 |
| **SAG5** | 13.3 | 15.03 | 19.01 | 16.01 | 15.01 | 0.99 |
| **SAG6** | 13.3 | 12.03 | 13.01 | 14.37 | 13.55 | 0.79 |

**S1-S5:** Experimental goat immunized with soluble antigen along with adjuvant; **C:** control (healthy adult goat).

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