Dept. of Microbiology,
Fac. of Vet. Med., Cairo University

PRELIMINARY STUDY ON THE PRODUCTION OF NITRIC OXIDE AND EXPRESSION OF NITRIC OXIDE SYNTHASE BY CAMEL MACROPHAGES

(With One Table and One Figure)

By

M. HASHAD; M. AL DUBAIB*; K. AL KHERIJI* and SAHER A.GALAL**

*Dept. of Vet. Medicine, College of Agriculture and Vet. Med., Qassim Univ.
**Dept. of Immunity, Amimal Health Research Institute, Dokki, Giza, Egypt.

(Received at 24/9/2006)

دراسة مبدئية لإنتاج أكسيد النيتريك والتعبير الجينى عن انزيم تمثيل أكسيد النيتريك لماكروفاج الجمال

محمود السيد حشاد ، محمود الدبيب ، خالد الخريجى
سحر عبد اللطيف جلال

من المعروف أن أكسيد النيتريك ينتج بواسطة الماكروفاج المحفزة بواسطة بعض الانتيجينات أو الميكروبات. فى هذه الدراسة تم فصل كرات الدم البيضاء من دم الجمال بواسطة الفيكول وبعد ذلك تم فصل الخلايا وحيدة النواة وهذه الخلايا تم إنمائها إلى الماكروفاج. تم تعـريض خلايا الماكروفاج البالغة على متعدد السكريات الدهنية المشتقة من الايشيرشيا كولاى وكـذا أنتيجين جدرى الأغنام وتم قياس كل من أكسيد النيتريك والحمض النووى المرسل الخـاص بإنزيم تمثيل أكسيد النيتريك وقد استطاع كلا الانتيجينين تحفيز إنتاج أكسيد النيتريك مع كون أنتيجين جدرى الأغنام الأقل تحفيزاً. وعند إجراء اختبـار تفاعـل البـوليميريز المتسلسـل بإستخدام بوادئ خاصة بالحمض النووى المرسل الخاص بإنتاج أكسيد النيتريك المنتج مـن ماكروفاج الجمال أسفرت الدراسة عن منتج بنفس حجم المقابل له فى الفئران مما يدل علـى ثبات فى العامل الوراثى الخاص بذلك.

SUMMARY

Nitric Oxide is produced by macrophage when activated or invaded by certain antigens or microbes. In the present study, camel peripheral blood leukocytes were obtained by ficoll cushion. Monocytes were separated and grown to macrophages. The mature macrophages were exposed to E. coli LPS as well as sheep pox virus antigen. Nitric oxide

(NO) production, by stimulated macrophages as well as the mRNA specific for production of inducible nitric oxide syntheses (iNOS), were investigated. Production of NO was stimulated by both pox antigen and LPS and was more with the latter. The obtained results indicated the similarity in the tested parameters between camel macrophages and those of other mammals tested so far viz: mice.

Key words: *Macrophages, camel, PCR, nitric oxide*

INTRODUCTION

Macrophages are major effectors against most bacterial infections, also transport bacteria between host tissues and provide a protected site for intracellular bacterial replication and it have different mediators for the microbicidal activities (Campbell, 1991; Tizard, 2000 and Okamura *et al.,* 2005). Reactive oxygen intermediates (ROls) are known as very powerful micrbicidal factors inside phagocytes (Kuby, 1994). Also, reactive nitrogen intermediates (RNls) are of equal importance for macrophages. When stimulated by factors such as bacterial cell wall LPS or gamma interferon (IFN-γ), macrophages begin to express high levels of nitric oxide synthase (NOS) which oxidizes L-arginine to yield citruline and a reactive radical, nitric oxide (NO), which has potent antimicrobial activity (Monacada and Higgs, 1993 and Matsuno *et al.,*1998) Most of the antimicrobial activities of macrophages against bacterial, fungal, helmenthic, and protozoal pathogens are suggested to be due to nitric oxide and its derivatives, where bacterial elimination occurs through phagocytosis and nitric oxide system (Franchini *et al,* 1995) this role has been recorded against Cryptococcus neoformans, Leishmania, Toxoplasma gondii, Trypanosoma cruzei, Listeria monocytogenes and Mycobacterium spp.(Gebran *et al.,* 1994). On the other hand, ROls have a major role in the control of intramacrophage killing of Brucella microorganisms while blocking of NO pathway resulted in minor levels of blocking macrophage antibrucella activities (Jiang *et al.,* 1993).

The role of NO as a microbicidal armature by camel macrophages is not investigated. The present study was planned to detect the production of NO by camel macrophages after non specific activation by bacterial or viral antigens. Also, conservation of iNOs mRNA in camel macrophages was aimed to be investigated by reverse transcription-polymerase chain reaction (RT-PCR) using mouse iNOS-specific oligonucleotides.

MATERIALS and METHODS

Macrophages:

Four hundred fifty millilitres of venous blood was collected by aseptic venipuncture from a healthy camel into a human blood donor bag. The blood was diluted with equal volume of phosphate buffered saline (PBS, pH 7.2). The leukocytes were separated by layering over ficoll cushions. After three washins in PBS, cells were suspended in RPMI-1640 tissue culture medium and adjusted to $5X10^6$ monocytes/ml and distributed into tissue culture polypropylene flasks. The monocytes were allowed to adhere and grow for 10 days at 37°C and 5% CO2 with 80% relative humidity (Campbell, 1991).

Activation and nitrogenous compound detection

Macrophages were prepared and activated using methods described by Jiang and Baldwin (1993) and Jiang *et al.* (1993). Macrophages were harvested by placement on ice back and fourth with gentle agitation. Cells were washed three times in PBS, suspended in RPMI-1640 (containing 10% autologous serum) and 100 µl distributed into wells of 96-well tissue culture plates (10^5 cells/well). *E. coli* LPS (200 ng/ ml RPMI) was added in 100 µl volumes into wells of a whole column. Also, 100 µl of sheep pox vaccine antigen were added to wells of another column. The plates were incubated at 37°C and 5% CO2 with 80% relative humidity for 24 hours after which nitrogenous compounds were estimated and estimated after 2 and 4 days also in the macrophage supernatants after reaction with Griess reagent (I% sulfanilamide, 0.1% naphtlylethylene diamine dihydrochloride, 2.5% H_3PO_4) according to the method of Gebran *et al.* (1994). This was done at a dual wavelength of 570/630 nm in an ELISA reader (Flow Laboratories, England).Results were analysed according to Bain and Engelhardt (1989).

Detection of iNOS production

This was performed according to the method described by Stenger *et al.* (1994). The total RNA was isolated from camel macrophages using the phenol extraction, reverse transcribed into cDNA by using reverse transcriptase (Amersham, Germany) and 1 µg of RNA. The cDNA was amplified by PCR using primers specific for human iNOS mRNA. Primers sequences were 5'- T AGAGGAACA TCTGGCCAGG-3' and 5' -AGGGTCCCCTCTGA TG-3' corresponding to positions 682-701 and 1052-1034 of human hepatocyte iNOS mRNA sequence. PCR amplification products were analyzed on 1.5% agarose gel containing ethidium bromide (0.5 mg/ml).

RESULTS

Table 1: Nitrate production levels by camel macrophages after LPS and sheep pox virus antigen activation.

Days post activate IOn	Non-activated Cells	LPS-activated Cells	Sheep Pox virus-activated cells
One day	3.52 ± 0.75*	27.82 ± 3.41	18.21 ± 2.80
Two days	3.83 ± 0.56	42.14 ± 4.20	20.34 ± 3.31
Four days	5.25 ± 0.86	36.11±3.01	22.16±4.83

* values = the mean of nitrogenous compounds in nanograms ± standard deviation
Differences between treatments were significant at $p<0.05$

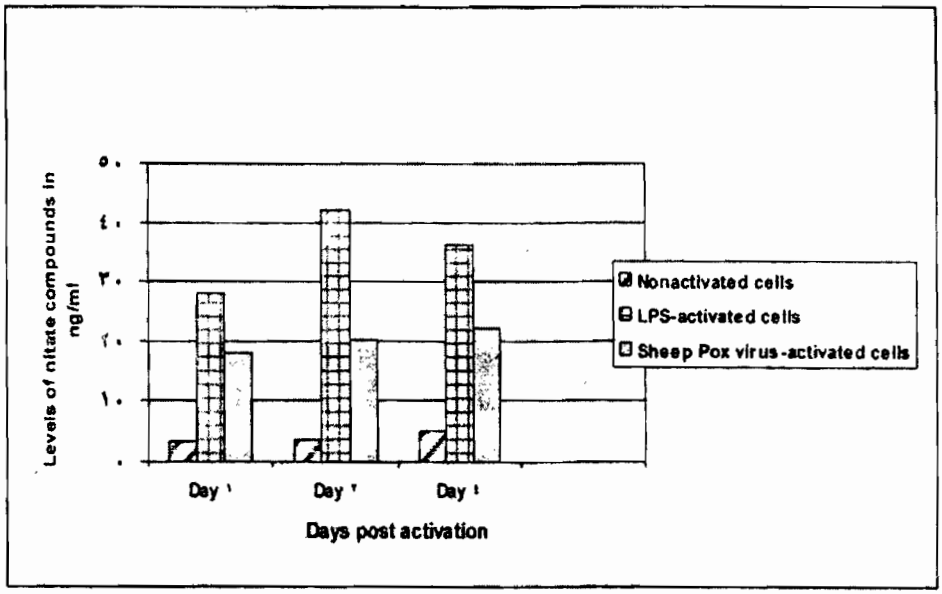

Fig. 1: Nitrogenous compounds produced by activated camel macrophages.

DISCUSSION

Most of the antimicrobial activities of macrophages against bacterial, fungal, helmenthic, and protozoal pathogens are suggested to be due to nitric oxide and its derivatives. Of the microorganisms that are affected by reactive nitrogen intermediates (RNIs) Cryptococcus neoformans, Leishmania, Toxoplasma gondii, Trypanosoma cruzei, Listeria monocytogenes and Mycobacterium spp. are the most common 0nes (Gebran *et al.*, 1994 and Gopinath *et al.*, 2006).

However, previous studies were conducted on phagocytes of mice, human and animals other than camels. Therefore, the present study was conducted to detect the expression of inducible nitric oxide synthase by activated camel macrophages. Also the production of nitrogenous compounds by camel macrophages should be investigated. Ficoll histopaque has been used to separate leukocytes from the whole blood of different origin (Hudson and Hay, 1991 and Franchini *et al.*, 1995).

In the present study, peripheral blood leukocytes of camels were successfully isolated using ficoll histopaque. Lipopolysaccharide (LPS)-activated macrophages produced high levels of nitric oxide and nitrogenous products at 24 and 48 hours and the level subsided at 96 hours postactivation (Table 1). This finding comes in agreement with the findings of Green *et al.*, (1991 and Matsuno *et al.*, 1998) who reported the role of nitric oxide as important trigger signal for nitric oxide synthesis by macrophages. The role of LPS was found to be early in the first two days and is likely a triggering factor for other inducers such as gamma interferon.

Concerning sheep pox antigen-activated macrophages, nitric oxide and nitrogenous compound intermediates were also detected in the cellular supernatants but in levels lower than those detected with the LPS. However, the activation persisted up to four days post activation and may be for a longer time without subsiding. This may be attributed to the nature of the antigen which is a living viral vaccine (Table 1 and Figure 1).

With the application of RT-PCR on RNA extracted from activated camel macrophages, human iNOS mRNA-specific primers succeeded in the amplification of the homologous sequence in camel. The same finding was recorded with murine macrophage mRNA (Chan *et al.*, 1994). Moreover, such method has been used to compare among the expression levels of mRNA of iNOS by macrophages from different animals (Kramnik *et al.*, 1993).

Conclusively, results obtained in this study indicate that nitric oxide and nitrogenous compound intermediates are produced by activated camel macrophages like macrophages of other animal species. This supports, to a considerable limit, the stability of the microbicidal armatures in different animal species. However, further studies are required to detect production of other armatures such as the active oxygen radicals by camel macrophages. Moreover, the iNOS mRNA was found to be conserved among the species studied so far including camel.

REFERENCES

Bain, L. and Engelhardt, M. (1989): Introduction to probability and mathematical statistics, PWS – Kent publishing Company.

Campbell, G.A. (1991): Application of monocyte-derived cell line to the study of phagocytic function in bovine brucellosis, Ph. D. Thesis, area of specialization "microbiology", Texas A&M University, CS, TX, 77843, USA.

Chan, J.; Tanaka, K.; Carroll, D.; Flynn, J. and Bloom, B.R. (1994): Effects of nitric oxide synthase inhibitors on murine infection with Mycobacterium tuberculosis, Infect. Immunol, 63(2): 736-740.

Franchini, A.; Fontanili, P. and Ottaviani, E. (1995): Invertebrate immunocytes: relationship between phagocytosis and nitric exde produetion, comporative Biochemistry and moleculear Biology, 110, (2) 403-407.

Gebran, S.J.; Yamamoto, Y.; Newton, T.; Klen, T.W. and Friedman, H. (1994): Inhibition of *Legionella pneumphila* growth by gamma interferon in permissive A/J mouse macrophages: Role of reactive oxygen species, nitric oxide, tryptophan, and iron, Infect. Immunology, 62(8): 3197-3205.

Gopinath, V.K.; Musa, M.; Samsudin, A.R; Lalitha, P. and Sosroseno, W. (2006): Role of Nitric oxide in hydroxyapatite-induced phagocytosis by murine macrophage cell line (RAW 264.7), Achives of oral Biology, 51- 339- 344.

Green, S.J.; Nacy, C.A. and Meltzer, M.S. (1991): Cytokine-induced synthesis of nitric oxides in macrophages: A protective host response to leishmania and other intracellular pathogens, 1. Leukocyte BioI., 50, 93-103.

Hudson, L. and Hay, F.C. (1991): Practical Immunology, Blackwell Scientific Publications, Oxford.

Jiang, X. and Baldwin, C.L. (1993): Effects of Cytokines on intercellular growth of Brucella abortus. Infect. Immun., 6 (10): 124–134.

Jiang, X.; Leonard, B.; Benson, R. and Baldwin, C.L. (1993): Macrophage control of Brucella abortus: Roie of reactive oxygen intermediates and nitric oxide, Cellular ImmunoI., 151, 309-319.

Kramnik, I.; Skamene, E. and Danuta, R. (1993): Assesment of lymphokine profiles in activated lymphocytes by semiquantitative PCR., J. Immunol. Methods, 162, 143-153.

Kuby, J. (1994): Immunology: second edition, W.H. Freeman and Company, New York, USA.

Matsuno, R.; Aramaki, Y.; Arima H.; Adachi, Y.; Ohno, N.; Yadomae, T. and Tsuchiya, S. (1998): Contribution of CR3 to Nitric oxide production from Macropnages stimulated with high-dose of LPS., Biochemical and Biophysical Research Communications, 244, Issue 1,6 march, 115-119.

Monacada, S. and Higgs, A. (1993): Mechanism_of disease. The L-arginine-nitric oxide pathway, The New. Eng. J. Med., 329(27): 2002-2012.

Okamura, M.; Lillehoj, H.S.; Raybourne, R.B.; Babu, U.S.; Heckert, R.A.; Tani, H.; Sasai, K.; Baba, E. and Lillehoj, E.P. (2005): Differential Responses of macrophages to Salmonella enterica serovars Enteridis and Typhimurium, Vet. Immun. and Immun. Path., 107-327-335.

Stenger, S.; Thuring, H.; Rollinghoff, M. and Bogdan, C. (1994): Tissue expression of inducible nitric oxide synthase is closely associated with resistance to Leishmania major. J. Exp. Med., 180, 783-793.

Tizard, I.R. (2000): Veterinary Immunology: An Introduction. 6th ed., W. B. Saunders Company., Philadelphia, Pennsylvania, USA.