PRODUCTION OF H_2O_2 AND NO BY RAT PERITONEAL MACROPHAGES IN RESPONSE TO GUT COMMENSAL BACTERIA

KOVAČEVIĆ-JOVANOVIĆ VESNA*, MITIĆ KATARINA*, STANOJEVIĆ STANISLAVA*,
MILETIĆ TATJANA*, VUJIĆ VESNA** and DIMITRIJEVIĆ MIRJANA*

*Immunology Research Center "Branislav Janković", Institute of Virology, Vaccines and Sera "Torlak", Belgrade, Serbia
**Institute of Chemistry, School of Medicine, University of Belgrade, Belgrade, Serbia

The importance of commensal bacteria in the immune system development and its involvement in the etiopatogenetic mechanisms of complex multifactorial and multigenic diseases is well documented. The aim of the present study was to compare the levels of hydrogen peroxide (H_2O_2) and nitric oxide (NO) produced by resident peritoneal macrophages from the autoimmune disease susceptible Dark Agouti (DA) rats vs. resistant Albino Oxford (AO) rat strain, under basal conditions and subsequent to in vitro stimulation with gut commensals. Following the stimulation with phorbol myristil acetate (PMA), E. coli/PMA or P. mirabilis/PMA, AO rats macrophages have produced significantly higher levels of H_2O_2 compared to the cells from DA rats. Strain differences in NO production were not detected under basal conditions and after the stimulation with lipopolysaccharide and P. mirabilis. However, after the in vitro stimulation with E. coli, AO rats macrophages have produced higher levels of NO compared to DA rats macrophages. Our results demonstrated that macrophages from AO rats have higher potential to produce H_2O_2 and NO in response to specific commensal bacteria when compared to DA rats. A possible relationship between the macrophage activity in response to commensal bacteria and the susceptibility to induction of autoimmune/inflammatory diseases in AO and DA rat strains is suggested.

Key words: commensal bacteria, hydrogen peroxide, peritoneal macrophages, nitric oxide, rat strains

INTRODUCTION

It is well established that two inbred rat strains, Dark Agouti (DA) and Albino Oxford (AO) differ in their sensitivity to induction of autoimmune inflammatory diseases. Namely, DA rats are sensitive and AO rats are resistant to experimental allergic encephalomyelitis (Mostarica-Stojković *et al*., 1982; Bouwer and Hinrichs,

1997; Dimitrijević *et al*., 2000) and arthritis (Dimitrijević *et al*., 2001). Multiple mechanisms are believed to control autoimmune disease resistance/sensitivity in the above mentioned strains. We have previously reported that reactive oxygen species (ROS) contribute to differences in susceptibility to experimental arthritis in DA and AO rat strains (Miletić *et al*., 2007).

Macrophages, known as phagocytic cells, are capable to produce ROS and nitric oxide (NO) (Babior, 1984). ROS include H_2O_2 and superoxide radicals, out of which other reactive oxidants such as oxidizing radicals and the oxidized halogens are produced (Babior, 1984). These reactive oxidants are produced for the purpose of killing invading microorganisms, but they also inflict damage of nearby tissues, and are thought to be of pathogenic significance in a large number of diseases (Babior, 2000). ROS are involved in many physiological processes including cell growth, differentiation and apoptosis (Hensley and Floyd, 2002). There is sufficient evidence for the critical role of ROS in the development of inflammatory processes and pathogenesis of numerous diseases, including rheumatoid arthritis (RA) (Hitchon and El-Gabalawy, 2004).

Another anti-microbial effector molecule, NO, has been found to play important roles in the organism: apart from its role as a signal molecule, it is also a cytotoxic and regulatory molecule of the innate immune response (Kroncke *et al*., 1998). However, the possible role of NO in the susceptibility to autoimmune inflammatory diseases is controversial. It is evident that NO has both beneficial and harmful roles during central nervous system autoimmune diseases (Dalton and Wittmer, 2005). Although NO may play a physiological role in lymphocyte cell signalling, its overproduction may perturb T cell activation, differentiation and effector responses, each of which may contribute in different ways to the pathogenesis of autoimmunity (Nagy *et al*., 2007).

Furthermore, the important role of commensal bacteria in the development of a normally functioning immune system is well known, as well as the involvement of commensal microflora in the etiopatogenesis of various complex multifactorial and multigenic diseases, including RA (Tlaskalova-Hogenova *et al*., 2004). For these reasons, the present study was set up to determine if there were any differences in H_2O_2 and NO production of resident peritoneal macrophages between DA and AO rat strains after their stimulation with commensal bacterial antigens. This approach is another attempt to provide further information about the contribution of genetic background to the susceptibility/resistance to induction of autoimmune diseases.

We have decided to use commensal bacterial antigens, *Escherichia coli* (*E. coli*) and *Proteus mirabilis* (*P. mirabilis*) that were the predominant Gram-negative aerobes in our rats' intestinal bacterial flora. Besides, the involvement of *E. coli* and *P. mirabilis* in RA pathogenesis has been suggested earlier (Tiwana *et al*., 1999). For instance, cross-reactivity between a peptide sequence EQKRAA of *E. coli* and RA susceptibility sequence has been demonstrated (Albani *et al*., 1992). Also, it has been reported that RA patients generate specific antibodies against *P. mirabilis* (Deighton *et al.*, 1992; Ebringer *et al.*, 1985; Wilson *et al*., 1995).

Acta Veterinaria (Beograd), Vol. 59. No. 2-3, 111-122, 2009.
Kovačević-Jovanović Vesna *et al*.: Production of H_2O_2 and NO by
rat peritoneal macrophages in response to gut commensal bacteria

113

MATERIALS AND METHODS

Animals

Six months old male rats of DA and AO strains, were used in the experiments. DA rats were derived from our breeding colony at the Immunology Research Center "Branislav Janković", Belgrade, while AO rats were obtained from the Military Medical Academy, Belgrade. Animals were housed in standard cages with free access to food pellets and tap water. All procedures involving animals and their care were approved by our Institutional Animal Care and Use Committee and followed principles described in the European Community's Council Directive of 24 November 1986 (86/609/EEC).

Bacterial antigens

Bacterial species were isolated from stool specimens collected from DA and AO rats. Fecal samples were collected from healthy adult rats and cultured using different agar plates (MacConcey agar, Endo agar) under aerobic conditions, at 37 ^{o}C for 24 hrs. Well-isolated colonies were selected at random from the predominant flora and identified using Gram staining and morphological characteristics, as well as biochemical tests and a rapid identification system (ID-32A, API Biomerieux). In DA rats *E. coli* and *P. mirabilis* were identified as the predominant aerobic flora, while in AO rats *E. coli* was isolated, but not *P. mirabilis*. Each of the species isolated was grown aerobically for 24 hrs. The bacteria were harvested at the stationary growth phase (24 hrs), washed with sterile phosphate-buffered saline (PBS), centrifuged at 8000 rpm for 20 minutes, and resuspended in PBS. The estimated number of bacteria in the suspension was determined by reading the optical density at 580 nm and extrapolating the value using a standard curve.

Resident Peritoneal Macrophages Harvesting

Peritoneal cells were harvested from DA and AO rats by washing the peritoneal cavity with sterile PBS. The cell suspensions were centrifuged at 1200 rpm for 10 minutes, washed once in sterile PBS and centrifuged again. The number of cells was adjusted under laminar flow to a density of 2.5×10^6/mL and 1×10^6/mL for H_2O_2 and NO assay, respectively. Resident peritoneal macrophages were purified by adherence to plastic on flat-bottom 96-well tissue culture plates (Nunc) for 2 h at 37 ^{o}C before the assay.

H_2O_2 assay

The present study aimed to determine H_2O_2 production levels by resident peritoneal macrophages from naive DA and AO rats after *in vitro* incubation of cells with RPMI, followed by additional stimulation with PMA. The effect of the whole cell *E. coli* and *P. mirabilis* stimulation, added prior to the incubation with PMA, on macrophage activity reflected in H_2O_2 production was also addressed. The amount of H_2O_2 released by macrophages was determined by reduction of nitro blue tetrazolium (NBT) salts to dark coloured formazans according to Pick *et al*. (1981). The cells were incubated for 24 h at 37 ^{o}C / 5% CO_2 with 100 μL/well:

114
Acta Veterinaria (Beograd), Vol. 59. No. 2-3, 111-122, 2009.
Kovačević-Jovanović Vesna *et al*.: Production of H_2O_2 and NO by
rat peritoneal macrophages in response to gut commensal bacteria

RPMI supplemented with 5% FCS (RPMI), 2×10^7 *E. coli*/RPMI or 2×10^7 *P. mirabilis*/RPMI. After the incubation period, the wells were extensively washed with sterile RPMI heated to 37 °C, and then 50 µL of RPMI or 50 µL of 50 nM of phorbol 12-myristate13- acetate (PMA, Sigma, St. Louis, Mo., USA) RPMI was added to wells, followed by 50 µL of 1 mg/ml NBT. The plates were incubated at 37 °C for 30 minutes, and then inverted and tapped onto clean paper tissue until dry. The cells were fixed by addition of 100 µL/well of methanol for 6 minutes and left to dry overnight. The optical density (OD) was measured at 545 nm and the results were expressed as $OD_{(545\,nm)} \times 1000$, or as stimulation index = $[OD_{(545\,nm)\,treatment}/OD_{(545\,nm)\,cRPMI}]$.

NO assay

The effect of lipopolysaccharide (LPS), *E. coli* and *P. mirabilis* stimulation on NO production was investigated in macrophages drawn from DA and AO rats. Cells were incubated with 100 µL of: RPMI, 2×10^7 *E. coli*/RPMI, 2×10^7 *P. mirabilis*/RPMI or 1 µg/mL LPS/RPMI for 48 h, at 37 °C / 5% CO_2. Nitrite concentrations in the culture medium were measured by a method based on Griess reaction (Green *et al*., 1982). Concentration of nitrite (µM) in the samples was calculated using 1-70 µM $NaNO_2$ as a standard. Results were expressed as µM NO_2^-, or as stimulation index = $[µM\ NO_2^-_{(treatment)}\ /\ µM\ NO_2^-_{(RPMI)}]$.

Statistical analysis

All biometric calculations (mean, S.E.) were performed using statistical packages SPSS 10.0. In order to determine the significance of differences between the independent groups one factor ANOVA was used followed by Fischer's post hoc test. Strain differences were tested by means of t-test. Differences are regarded as statistically significant if $P < 0.05$. Results are presented as Mean ± S.E.

RESULTS

H_2O_2 production

In both rat strains H_2O_2 production was increased after the *in vitro* incubation of macrophages with PMA, in comparison with RPMI control (Figure 1A and 1B). When macrophages in the culture were additionally stimulated with *E. coli* antigens, the levels of H_2O_2 production were significantly higher in both rat strains in comparison with respective cells stimulated with PMA only. On the other hand, *P. mirabilis* increased PMA-stimulated H_2O_2 production only in macrophages from DA rats (Fig. 1A). Strain differences in H_2O_2 production were not evident after the *in vitro* incubation of macrophages with RPMI only (Figure 1A and 1B). However, when cultured macrophages were additionally stimulated with PMA, strain differences were noted: H_2O_2 production stimulation index was significantly higher in macrophages isolated from AO rats when compared to the corresponding group of DA rats (Figure 2). In addition, H_2O_2 production stimulation index was elevated in macrophages derived from AO rats after

Acta Veterinaria (Beograd), Vol. 59. No. 2-3, 111-122, 2009.
Kovačević-Jovanović Vesna *et al*.: Production of H_2O_2 and NO by
rat peritoneal macrophages in response to gut commensal bacteria

115

incubation with *E. coli*/PMA in comparison with the corresponding group of DA rats. Similar, but less pronounced, strain differences were obtained after macrophage stimulation with *P. mirabilis*/PMA.
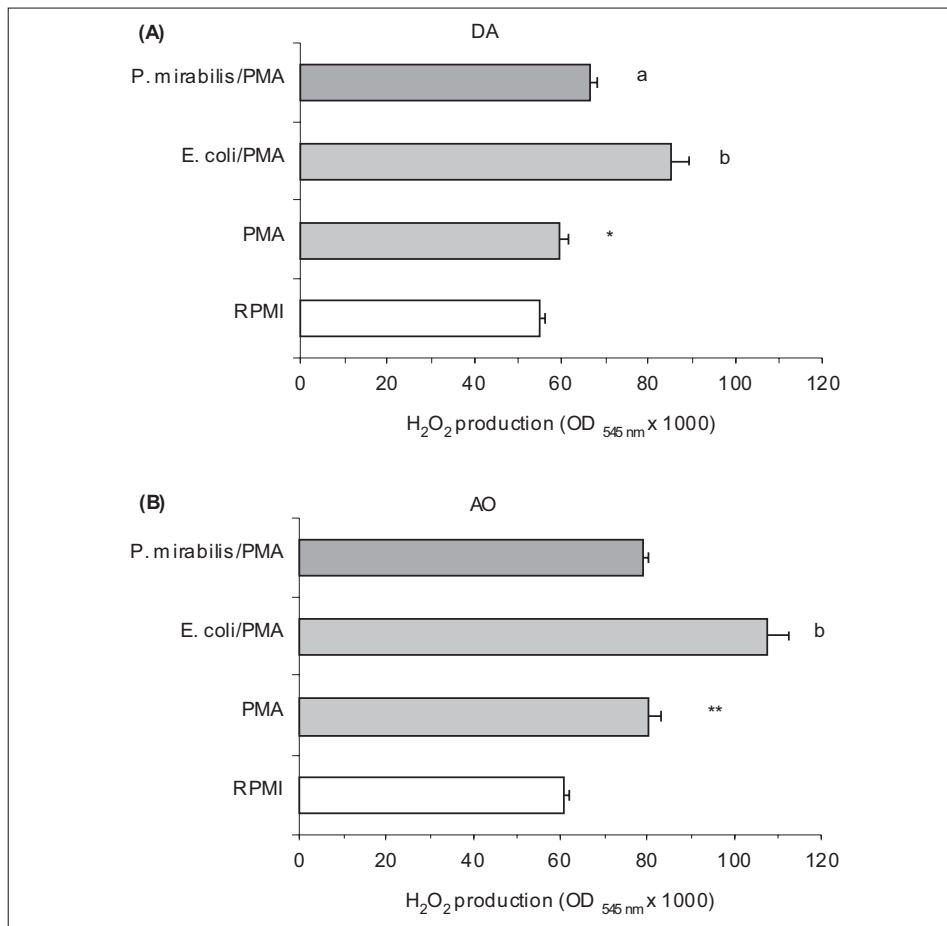

Figure 1. H_2O_2 production by resident peritoneal macrophages derived from DA (A) and AO rats (B). The cells were *in vitro* incubated for 24h with RPMI, 2×10^7 *E. coli* or 2×10^7 *P. mirabilis* in RPMI, followed by 30 min of incubation with 25 nM PMA. Control cells were incubated with RPMI only. The results are expressed as $OD_{545} \times 1000$ + S.E. Statistically significant differences: *$P<0.05$, and **$P<0.001$, vs. corresponding RPMI, and [a]$P<0.05$, and [b]$P<0.0001$ vs. corresponding PMA

116
Acta Veterinaria (Beograd), Vol. 59. No. 2-3, 111-122, 2009.
Kovačević-Jovanović Vesna *et al*.: Production of H_2O_2 and NO by
rat peritoneal macrophages in response to gut commensal bacteria
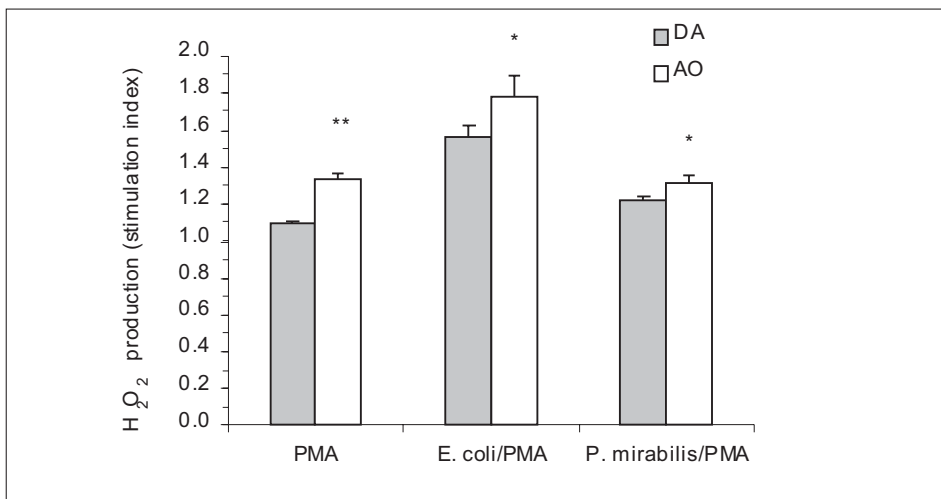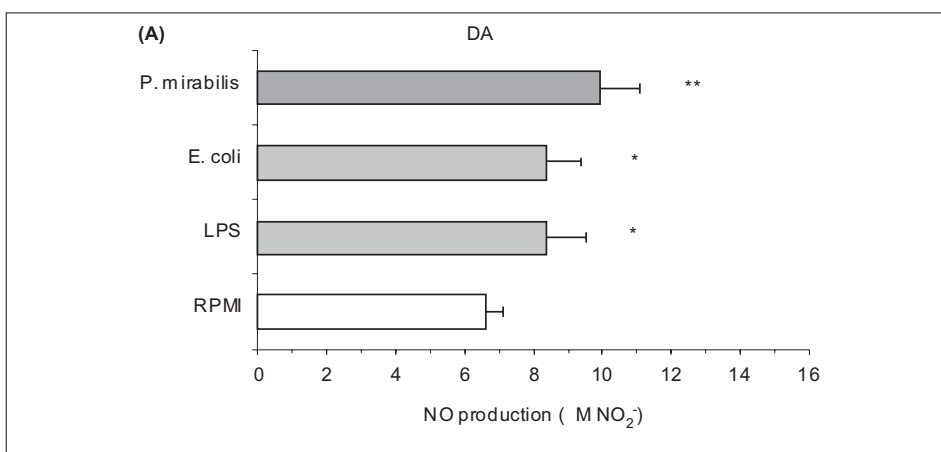
Figure 2. The differences in H_2O_2 production by resident peritoneal macrophages derived from DA and AO rats. The cells were *in vitro* incubated for 24h with RPMI, 2 x 10^7 *E. coli* or 2 x 10^7 *P. mirabilis* in RPMI, followed by 30 min of incubation with 25 nM PMA. The results are expressed as stimulation index + S.E. Statistically significant differences: *P<0.05, and **P<0.0001, AO vs. DA

NO production

Macrophages derived from both DA and AO rats produced significantly higher concentrations of NO when stimulated with LPS, *E. coli* and *P. mirabilis* in comparison with the RPMI control group (Figure 3A and 3B). Strain differences in NO production were not noted among experimental animals under basal conditions (Figure 3A and 3B), as well as after co-stimulation with LPS *in vitro* (Figure 4). However, significantly higher NO production stimulation index after

Acta Veterinaria (Beograd), Vol. 59. No. 2-3, 111-122, 2009.   117
Kovačević-Jovanović Vesna *et al*.: Production of H_2O_2 and NO by
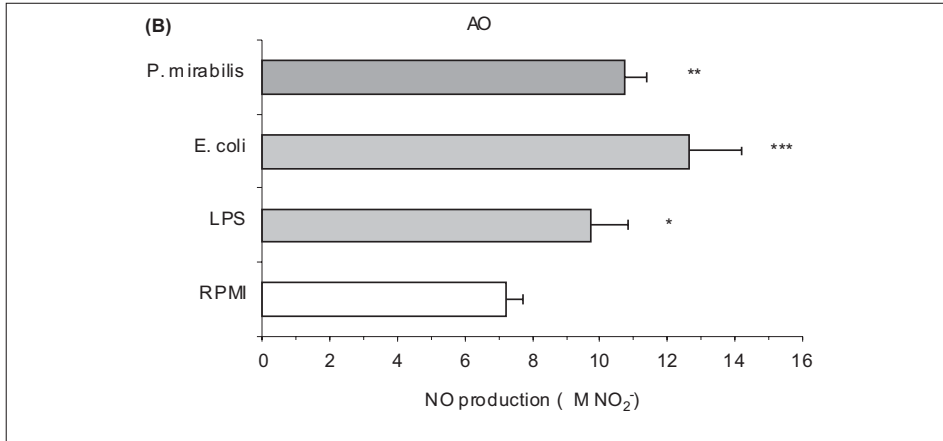rat peritoneal macrophages in response to gut commensal bacteria

Figure 3. NO production by resident peritoneal macrophages derived from DA (A) and AO
(B) rats. The cells were *in vitro* incubated with RPMI, 2×10^7 *E. coli*, 2×10^7 *P. mirabilis*
in RPMI, or 1 µg/mL LPS/RPMI for 48h. Control cell were incubated with RPMI only.
The results are expressed as µmol NO_2^- + S.E. Statistically significant differences:
*P<0.05, **P<0.01, ***P<0.001, vs. RPMI

incubation with *E. coli* was obtained in macrophages derived from AO rats in
comparison with macrophages derived from DA rats. On the other hand, no strain
differences were observed in NO production following the stimulation of cells with
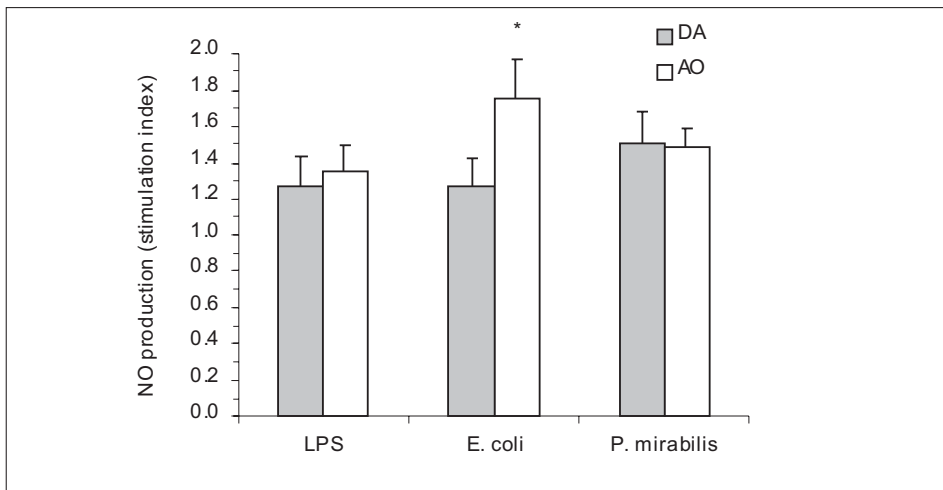*P. mirabilis*.

Figure 4. Differences in NO production by resident peritoneal macrophages derived from
DA and AO rats. The cells were *in vitro* incubated with RPMI, 2×10^7 *E. coli*, 2×10^7 *P.
mirabilis* in RPMI, or 1 µg/mL LPS/RPMI for 48h. The results are expressed as
stimulation index + S.E. Statistically significant differences: *P<0.05 AO vs. DA

118   **Acta Veterinaria (Beograd), Vol. 59. No. 2-3, 111-122, 2009.**
Kovačević-Jovanović Vesna *et al*.: Production of H_2O_2 and NO by
rat peritoneal macrophages in response to gut commensal bacteria

DISCUSSION

It is well documented that generation of H_2O_2 and the expression of NO-synthetizing enzyme inducible NO synthase (iNOS) by macrophages both occur during phagocytosis of bacteria, fungi and protozoa (Darrah *et al*., 2000; Miyagi *et al*., 1997; Pacelli *et al*., 1995). A strong macrophage activator, PMA, is also a known inducer of H_2O_2 generation. Also, the rapid generation of H_2O_2 is a cardinal feature of the cellular response of phagocytes to commensal bacteria (Lambeth, 2004). Therefore, our finding that commensal bacterial antigens increased H_2O_2 production is in line with these results. On the other hand, it is known that a spontaneous bacterial translocation occurs continuously at a low rate in a healthy host. Bacterial translocation is the passage of viable bacteria from the gastrointestinal tract to extraintestinal sites, including the mesenteric lymph nodes, liver, spleen and other end-organs (Berg, 1999). Shanahan *et al.* (2002) have shown this spontaneous translocation of bacteria to occur from the intestinal lumen to the mesenteric lymph nodes continuously in a healthy host. These migrating bacteria are generally efficiently killed, but the process may be of a great importance for the host by stimulating protective immunity. Peritoneal macrophages were chosen for this study, as they best represent the systemic macrophage activity. It may be speculated that bacterial cells might influence other macrophages in a similar way in which they influence peritoneal macrophages, by encountering them during their migration. Therefore, the interaction between the commensal microflora and macrophages may be one of the means of increasing H_2O_2 concentrations in the organism.

In our previous study we reported that the macrophages of the arthritis resistant strain (AO), exhibited a significantly higher respiratory burst compared to the arthritis susceptible strain (DA), following their immunization with incomplete Freund's adjuvant, pointing out to a possible role of reactive oxygen species in oil induced arthritis (Miletić *et al*., 2007). It may be argued that reactive oxygen species contribute to the resistance to adjuvant arthritis induction in AO rat strain in the same way (Miletić *et al*., 2007).

A body of evidence points out to the possible regulatory role of ROS in the immune response. Among others, a strong emphasis has been put on the role of ROS in cell signalling. It was shown that macrophages producing ROS were able to suppress the IL-2 production and T cell proliferation *in vitro* (Gelderman *et al.*, 2007). Aside from the observation that ROS affect T cells activation, it was shown that the type of T cell response is also influenced by ROS. King *et al*. (2006) found that when human T cells were stimulated *in vitro* with anti-CD3 and anti-CD28 in the presence of ROS, a clear Th2 phenotype development was observed in spite of the presence of Th1 cytokines (King *et al*., 2006). In addition, this less pronounced Th1 response in the presence of ROS was mediated via macrophages and resulted in decreased arthritis severity (Gelderman *et al*., 2007). Same authors hypothesized that, by producing ROS, macrophages in the central or peripheral lymphoid organs educate T cells to render them less activated in an antigen-dependent fashion in order to prevent autoimmune responses in the periphery or to downregulate an ongoing immune response. In

Acta Veterinaria (Beograd), Vol. 59. No. 2-3, 111-122, 2009.   119
Kovačević-Jovanović Vesna *et al*.: Production of H$_2$O$_2$ and NO by
rat peritoneal macrophages in response to gut commensal bacteria

the light of these findings, the decreased capacity to produce ROS by DA macrophages and the increased oxidation status in AO rats observed in our study, might at least partially contribute to different autoimmune disease susceptibility of these two strains.

The NO generated by iNOS, acts as both an immunoregulatory and effector molecule in numerous inflammatory and autoimmune diseases or their experimental models (Hensley and Floyd, 2002). The NO metabolic pathway is a key defense process against viruses and tumors. NO is also involved in T-cell immunosupression induction during infection, as well as in the killing of microorganisms (Nascimento *et al*., 2002). Furthermore, the differences in NO macrophage production were associated with resistance or susceptibility to infection with certain microorganisms (Nascimento *et al*., 2002).

In the present study no significant strain differences in NO production between DA and AO peritoneal cells, both under basal conditions and following their stimulation with LPS and *P. mirabilis*, were noted. On the other hand, the increased levels of NO were detected in macrophages derived from AO rats after their *in vitro* incubation with *E. coli,* compared to the corresponding experimental group of DA rats. LPS, a component of the outer membrane of Gram-negative bacteria, which initiates cellular inflammatory responses (Elder *et al.*, 2000), is one of the most potent activators of macrophages and a known inductor of iNOS and NO secretion (Klimp *et al*., 2001). In our experiments, LPS has lead to the same level of increase of NO production in both rat strains. It is interesting to note that the stimulation of cells with commensal bacteria, *E. coli,* has lead to a significantly higher increase of NO production in AO rats. This could be due to two possible causes: either the two isolated *E. coli* strains used encompass the equivalent biochemical properties, but their other features (e.g. the amount of LPS in their cell wall, its composition and distribution, etc.) might be quite different; or this finding cannot only be explained by the presence of LPS in *E. coli* membrane. It is still unclear if a similar increase of NO production could also occur *in vivo*, for example, due to the migration of commensal bacteria.

Anyhow, the role of NO as the immunoregulatory molecule is still a subject of intensive research. Recent studies revealed that NO is not essential for the resistance of AO rats to EAE (Miljković *et al.*, 2006). The results from our laboratory have shown no causal relationship between the amount of NO produced by peritoneal macrophages and the AA/OIA susceptibility and severity (Miletić *et al*., 2007).

In the healthy host, there is sufficient evidence that NO may directly alter T lymphocyte function in different ways. NO promotes mytochondrial hyperpolarization, ATP depletion and relative resistance to apoptotic stimuli in lymphocytes (Beltran *et al.*, 2002). NO appears to be a bifunctional modulator of cell death capable of either stimulating or inhibiting apoptosis (Kim *et al*., 1999). Also, NO is a key signalling intermediate in T cell activation (Nagy *et al.*, 2003; 2007). As a highly diffusible gas, NO produced by neighboring cells may affect the T cell function either directly by post-translational modification, by regulating gene expression, or mitochondrial biogenesis or indirectly through depletion of L-arginine (Nagy *et al.*, 2007). Results that macrophages from AO rats produced a

120   **Acta Veterinaria (Beograd), Vol. 59. No. 2-3, 111-122, 2009.**
Kovačević-Jovanović Vesna *et al*.: Production of H_2O_2 and NO by
rat peritoneal macrophages in response to gut commensal bacteria

higher level of NO encountering commensal bacteria, may rather be viewed as low production in DA rats, instead of an overproduction of NO in AO rats. Having in mind that NO regulates immune function in health, a decreased capacity to produce NO in response to the specific commensal bacteria by DA macrophages could provide a reducing enviroment required for optimal T cell proliferation. This, combined with the genetic predisposition, might contribute to the established autoimmune disease susceptibility of this rat strain.

In conclusion, we have shown that macrophages from AO rats have a higher potential to produce H_2O_2 and NO when stimulated *in vitro* with specific commensal bacterial antigens. Future studies will be undertaken to determine the biological significance of this elevated cell activity in AO rats. Certainly, explaining the differences in production of both pro-inflammatory mediators, H_2O_2 and NO, after the interaction with such a complex ecosystem as gastrointestinal flora will contribute to understanding the nature of commensal bacteria-host interactions.

Address fof correspondence:
Kovačević-Jovanović Vesna
Immunology Research Center "Branislav Janković"
Institute of Virology, Vaccines and Sera "Torlak"
11121 Belgrade, Serbia
Vojvode Stepe 458
E-mail: vkovacevic@torlakinstitut.com

REFERENCES

1. *Albani S, Tuckwell J, Esparza D, Carson D, Roudier J*, 1992, The susceptibility sequence to rheumatoid arthritis is a cross-reactive B-cell epitope shared by Escherichia coli heat shock protein dnaJ and the histocompatibility leucocyte antigen DRB10401 molecule. *J Clin Investig,* 89, 327-31.
2. *Babior BM*, 1984, Oxidants from phagocytes: agents of defence and destruction. *Blood*, 64, 959-66.
3. *Babior BM*, 2000, Phagocytes and oxidative stress, *Am J Med*, 109, 33-44
4. *Berg RD*, 1999, Bacterial translocations from gastrointestinal tract. *Advances in Experimental Medicine and Biology*, 473, 11-30.
5. *Beltrán B, Quintero M, García-Zaragozá E, O'Connor E, Esplugues JV, Moncada* S, 2002, Inhibition of mitochondrial respiration by endogenous nitric oxide: a critical step in Fas signaling, *Proc Natl Acad Sci U S A*, 99, 8892-7.
6. *Bouwer HG, Hinrichs DJ,* 1997, Experimental allergic encephalomyelitis and vaccination-induced resistance in DA rats, *Cell Immunol*, 175, 92-8.
7. *Bogdan C*, 2001, Nitric oxide and the immune response, *Nat Immunol*, 2, 907-16.
8. *Dalton DK, Wittmer M,* 2005, Nitric-oxide-dependent and independent mechanisms of protection from CNS inflammation during Th1-mediated autoimmunity: evidence from EAE in iNOS KO mice, *J Neuroimmunol*, 160, 110-21.
9. *Darrah PA, Hondalus MK, Chen Q, Ischiropoulos H, Mosser DM*, 2000, Cooperation between reactive oxygen and nitrogen intermediates in killing of *Rhodococcus equi* by activated macrophages. *Infect Immun*, 68, 3587-93.
10. *Deighton CM, Gray J, Bint AJ, Walker DJ*, 1992, Specificity of the proteus antibody response in rheumatoid arthritis, *Ann Rheum Dis*, 51, 1206-7.

Acta Veterinaria (Beograd), Vol. 59. No. 2-3, 111-122, 2009.   121
Kovačević-Jovanović Vesna *et al*.: Production of H_2O_2 and NO by
rat peritoneal macrophages in response to gut commensal bacteria

11. *Dimitrijević M, Laban O, Djurić VJ, Stanojević S, Miletić T, Kovacevic-Jovanović V et al.*, 2001, Behavior and severity of adjuvant arthritis in four rat strains, *Brain Behav Immun*, 15, 255-65.

12. *Dimitrijević M, Laban O, Stanojević S, Radulović J*, 2000, Induction and reinduction of experimental allergic encephalomyelitis in DA rats: the effect of age and encephalitogen, *Acta Vet Beograd* 50, 289-96.

13. *Ebringer A, Ptaszynska T, Corbett M, Wilson C, Macafee Y, Avakian H et al.*, 1985, Antibodies to proteus in rheumatoid arthritis, *Lancet*, 2, 305-7.

14. *Elder AC, Finkelstein J, Johnston C, Gelein R, Oberdorster, G*, 2000, Induction of adaptation to inhaled lipopolysaccharides in young and old rats and mice*, Inhal Tox*, 12, 225-43.

15. *Gelderman KA, Hultqvist M, Pizzolla A, Zhao M, Nandakumar KS, Mattsson R et al*, 2007, Macrophages suppress T cell responses and arthritis development in mice by producing reactive oxygen species, *J Clin Invest*, 117, 3020-8.

16. *Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR*, 1982, Analysis of nitrate, nitrite, and (^{15}N) nitrate in biological fluids. *Anal Biochem*, 126, 131-8.

17. *Hensley KF, Floyd R*, 2002, Reactive oxygen species and protein oxidation in ageing: a look back, a look ahead, *Arch Biochem Biophys*, 397, 377-83.

18. *Hitchon CA, El-Gabalawy HS*, 2004, Oxidation in rheumatoid arthritis, *Arthritis Res Ther*, 6, 265-78.

19. *Kim YM, Bombeck CA, Billiar TR*, 1999, Nitric oxide as a bifunctional regulator of apoptosis, *Circ Res*, 84, 253-6.

20. *King MR, Ismail AS, Davis LS, Karp DR*, 2006, Oxidative stress promotes polarization of human T cell differentiation toward a T helper 2 phenotype, *J Immunol*, 176, 2765-72.

21. *Klimp AH, de Vries EGE, Scherphof GL, Daemen T*, 2001, A potential role of macrophage activation in the treatment of cancer, *Crit Rev Oncol Hematol*, 44, 143-61.

22. *Kroncke KD, Fehsel K, Kolb-Bachofen V*, 1998, Inducible nitric oxide synthase in human diseases, *Clin Exp Immunol*, 113, 147-56.

23. *Lambeth JD*, 2004, NOX enzymes and the biology of reactive oxygen, *Nat Rev Immunol*, 4, 181-9.

24. *Miletić T, Kovacević-Jovanović V, Vujić V, Stanojević S, Mitić K, Lazarević-Macanović M et al.*, 2007, Reactive oxygen species (ROS), but not nitric oxide (NO), contribute to strain differences in the susceptibility to experimental arthritis in rats, *Immunobiol*, 212, 95-105.

25. *Miyagi K, Kawakami K, Saito A*, 1997, Role of reactive nitrogen and oxygen intermediates in gamma interferon-stimulated murine macrophage bactericidal activity against *Burkholderia pseudomallei*, *Infect Immun*, 65, 4108-13.

26. *Miljkovic D, Stosic-Grujicic S, Markovic M, Momcilovic M, Ramic Z, Maksimovic-Ivanic D, Mijatovic S et al.*, 2006, Strain difference in susceptibility to experimental autoimmune encephalomyelitis between Albino Oxford and Dark Agouti rats correlates with disparity in production of IL-17, but not nitric oxide, *J Neurosci Res*, 84, 379-88.

27. *Mostarica-Stojković M, Petrović M, Lukić ML*, 1982, Resistance to the induction of EAE in AO rats: its prevention by the pre-treatment with cyclophosphamide or low dose of irradiation. *Clin Exp Immunol*, 50, 311-7.

28. *Nagy G, Clark JM, Buzás EI, Gorman CL, Cope AP*, 2007, Nitric oxide, chronic inflammation and autoimmunity. *Immunol Lett*, 111, 1-5.

29. *Nagy G, Koncz A, Perl A*, 2003, T Cell Activation-Induced Mitochondrial Hyperpolarization Is Mediated by Ca^{2+}- and Redox-Dependent Production of Nitric Oxide, *J Immunol*, 171, 5188-97.

30. *Nascimento FR, Calich VL, Rodriguez D, Russo M*, 2002, Dual role for nitric oxide in paracoccidiomycosis: essential for resistance, but overproduction associated with susceptibility, *J Immunol,* 168, 4593-600.

31. *Pacelli R, Wink DA, Cook JA, Krishna MC, DeGraff W, Friedman N et al*, 1995, Nitric oxide potentiates hydrogen peroxide-induced killing of Escherichia coli. *J Exp Med*, 182, 1469-79.

32. *Shanahan F*, 2002, The host-microbe interface within the gut, *Best Pract Res Clin Gastroenterol*, 16, 915-31.

33. *Tlaskalová-Hogenová H, Stepánková R, Hudcovic T, Tucková L, Cukrowska B, Lodinová-Zádníková R et al.*, 2004, Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases, *Immunol Lett*, 93, 97-108.

34. *Tiwana H, Wilson C, Alvarez A, Abuknesha R, Bansal S, Ebringer A,* 1999, Cross-reactivity between the Rheumatoid Arthritis-Associated Motif EQKRAA and Structurally related Sequences Found in Proteus mirabilis, *Infect Immun*, 67, 2769-75.
35. *Wilson CA, Ebringer A, Ahmadi K, Wrigglesworth J, Tiwana H, Fielder M et al.,* 1995, Shared amino acid sequences between major histocompatibility complex class II glycoproteins, type XI collagen and Proteus mirabilis in rheumatoid arthritis, *Ann Rheum Dis*, 54, 216-20.

PRODUKCIJA H_2O_2 I NO PERITONEALNIH MAKROFAGA PACOVA U ODGOVORU NA CREVNE KOMENSALNE BAKTERIJE

KOVAČEVIĆ-JOVANOVIĆ VESNA, MITIĆ KATARINA, STANOJEVIĆ STANISLAVA, MILETIĆ TATJANA, VUJIĆ VESNA i DIMITRIJEVIĆ MIRJANA

SADRŽAJ

Poznato je da komensalna crevna flora ima značajnu ulogu u razvoju imunskog sistema kao i u etiopatogenezi kompleksnih multifaktorijalnih i multigenetskih bolesti. Cilj ovog rada bio je da se uporedi produkcija vodonik peroksida (H_2O_2) i azot monoksida (NO) peritonealnih makrofaga dva inbredna soja pacova, od kojih je jedan osetljiv (Dark Agouti, DA), a drugi rezistentan (Albino Oxford, AO) na indukciju autoimunskih bolesti, kako u bazalnim uslovima tako i nakon *in vitro* stimulacije makrofaga sa crevnim komensalima. Nakon stimulacije sa forbol miristil acetatom (PMA), *E. coli*/PMA and *P. mirabilis*/PMA makrofage AO pacova su produkovale značajno više H_2O_2 u poređenju sa makrofagama DA pacova. Nisu detektovane sojne razlike u produkciji NO u bazalnim uslovima, kao ni posle stimulacije sa lipopolisaharidom i *P. mirabilis*. Međutim, nakon *in vitro* stimulacije sa *E. coli* makrofage AO pacova su produkovale više NO u odnosu na makrofage DA pacova. Naši rezultati su ukazali da makrofage AO pacova imaju veći potencijal za produkciju H_2O_2 i NO u odgovoru na specifične komensalne bakterije. Ova različita aktivnost makrofaga može biti u vezi sa različitom osetljivošću na indukciju autoimunskih/inflamatornih bolesti kod DA i AO soja pacova.