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PRODUCTION OF REACTIVE OXYGEN (H_2O_2) AND NITROGEN (NO) INTERMEDIATES AND TNF-α IN MICE GENETICALLY SELECTED FOR HIGH (H) AND LOW (L) ANTIBODY RESPONSE AND EXPERIMENTALLY INFECTED WITH *LEPTOSPIRA* SEROVAR POMONA

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ABSTRACT

The aim of the present study was to evaluate the activity of macrophages, and the production of TNF-α and antibodies against experimental infection by *Leptospira* serovar Pomona in mice genetically selected for High (H) or Low (L) humoral immune response. To evaluate macrophagic activity, peritoneal and splenic lavages were performed for determination of oxygen (H_2O_2) and nitrogen (NO) intermediates. The production of the tumor necrosis factor (TNF-α) was investigated through bioassays in serum and homogenates of splenic and hepatic cells of control and infected animals, as was as specific antibodies production. The immune response against serovar Pomona in those lines, was characterized by high antibody production, especially in later periods of the infectious process, whereas values of bacterial recovery in culture medium were lower. The production of reactives oxygen and nitrogen intermediate, also helped to eliminate *Leptospira* Pomona in both lines; H_2O_2 production an important factor in $H_{IV\text{-}A,}$ as well as NO production in $L_{IV\text{-}A}$, especially in later post-inoculation periods. The same was detected for TNF-α. Results suggest that such lines could be an important model to investigate the pathogenesis and the immune response of animals against the several *Leptospira* serovars.

Keywords: Immunological aspects; Biozzi mice; *Leptospira*; Macrophages; Cytokines.

INTRODUCTION

Leptospirosis is a widespread zoonotic infection of results ranging from subclinical infection, to a fatal presentation named Weil's disease. Despite the overall impact of this disease, the mechanisms of pathogenicity, host defense and protective immunity of *Leptospira* still remain unclear, especially for those related to the production of mediated immune response for the infection control, which is not similar in all hosts and depends on the infective serovar (8).

Protection against the several *Leptospira* serovars involves different factors such as specific antibodies

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and the complement system. However, the phagocytic activity of macrophages and granulocytes also seems to be efficient against leptospirosis. Thus, phagocytic cells actively participate in the inflammatory process, even in the absence of specific antibodies (12, 16, 28).

Activated macrophages have microbicidal properties through the action of lysosomal enzymes, release of cytokines such as TNF-α and IFN-γ, and reactive products of nitrogen and oxygen (2).

In guinea pigs infected with *Leptospira* serovar Icterohaemorrhagiae, there was a high production of the TNF-α relative to controls (20). In hamsters infected with *Leptospira* serovar Pyrogenes, TNF-α expression was identified in renal tissues through real-time PCR from the third day post-infection (18).

Initially from albino mice colonies outbred, Biozzi *et al* (5) other lines were developed and they were genetic selected for the study of the characterization of the mechanisms of regulation that are involved in the immune response. After this study, other studies have been done that generated the development of five selections (I to V).

This model has been obtain based on the bidirectional genetic selection of mice that had high response (H) and the ones that had a low response (L) in terms of production of antibodies against complex natural antigens for a period of 15 to 20 generations, until they have reached the highest divergence in the immune humoral response. The selection has modified the response not only of the selecting antigen but also of others antigens not really related with this process (10). The high and the low capacity of response are result of additional effect of alleles localized in different independent loci (polygenic control). Which have been accumulated progressively in the groups H and L during the selection process. These homozygotic lines represent extreme phenotypes that can be found in natural heterogeneous populations.

In the present study, genetically selected mice lines (selection IV-A) were used for the quantitative production of antibodies (6, 15). as well as changes in the metabolic activity of macrophages. This two lines of mice, high line (H_{IV-A}) and low line (L_{IV-A}) were genetically selected for their respective high and low antibody formation after immunization with sheep erythrocytes. The extensive quantitative difference between them involves not only all classes of immunoglobulin but also antibody synthesis in response to a wide range of unrelated antigens, cytodynamics of their B cells response and in the macrophage functions, namely those concerning the metabolism and presentation of the antigen to lymphocytes (6).

Mice were experimentally infected with *Leptospira* serovar Pomona in order to evaluate antibody production levels and peritoneal and spleenic macrophages activity through quantification of oxygen (H_2O_2) and nitrogen (NO) reactive intermediates and TNF-α production in serum and homogenates of splenic and hepatic cells of control and infected animals.

MATERIAL AND METHODS

Mice and Inoculum

In this study have been used thirty male mice of the IV-A Selection for high humoral response (H_{IV-A}) and other 30 mice of the IV-A selection for low humoral response (L_{IV-A}) with 4-6 weeks of age developed at the Immunology Lab of São Paulo Biological Institute and they were maintain in the Department of Microbiology and Immunology, Biosciences Institute, São Paulo State University (UNESP).

Leptospira strains were obtained from the Bacterial Zoonoses Lab, University of São Paulo (USP). Virulence of *Leptospira interrogans* serovar Pomona strain Fromm was maintained by iterative passages in Golden Syrian hamsters.

The mice were inoculated intraperitoneally with 1 mL inoculum containing $2x10^7$ leptospires identified through agglutinin-absorption tests (27) and quantified according to the method described by Faine, (11). Twenty-four negative

controls were also used. Before sacrifice, mice were anesthetized by light ether inhalation and partially bled by orbital plexus puncture to obtain serum for the in microscopic agglutination test. The animals were sacrificed at days 4, 7, 14, 21, 28 and 35 post-inoculation under the supervision of the Ethics Committee of Botucatu Medical College, São Paulo State University, Brazil, process 383/2004.

Recovery of leptospires

To assess the degree of infection by leptospires, fragments of kidneys and liver from the infected animals were cultured and isolated through the by serial dilution technique, according to Santa Rosa (27) and Passos *et al* (23).

Antibody titration

Anti-*Leptospira interrogans* serovar Pomona antibodies were detected in sera through microscopic agglutination test on slides according to the method described by Faine (11). Pomona and 24 other serovars were used to exclude mice previously infected or containing a different serovar.

Macrophage activity

After killing the animals, they were maintained in an aseptic laminar flow hood and they were fixed in a lying back position for dissection. The abdominal wall was exposed taking the skin out from that region. After that a cold solution of 10ml of PBS (Buffer solution phosphate pH 7,2) was inject in central superior abdominal region. Subsequently, the abdominal cavity was massaged and liquid was taken with needle from the peritoneal cavity. This procedure was done three times. Then, samples from spleen were taken and homogenised with PBS to obtain suspensions. The abdominal suspension and spleen suspension were kept in plastic tubes, maintain on ice and centrifuge after being collect for 10 minutes at 1500 rpm.

After centrifugation, the cellular suspension was maintained in complete medium for cell culture (MCCC). From each peritoneal suspension 50 µl aliquots were made and were supplemented with a solution that contained 0,45ml of neutral red at 0,02% and incubate at 37ºC for 10 minutes for a subsequent counting. The macrophages were identified by the incorporation of the neutral red and counted Neubauer chamber and the cellular concentration was adjusted to $2X10^6$ macrophages/ml of MCCC. Small volumes of 0,1ml of suspension were distributed in micro plates (Corning plates) for cell culture. After an incubation period of 2 hours at 37ºC with 5% of CO2, the non-adherents cells were taken using a wash with RPMI 1640 medium.

Hydrogen peroxide production

After obtaining macrophages by peritoneal lavages and splenic tissue suspensions, of hydrogen peroxide (H_2O_2) production was quantified through the method described by Pick & Keisari (24) and adapted by Pick & Mizel (25). H_2O_2 production was investigated without stimuli or with the addition of interferon gamma (IFN-γ) and Phorbol-Myristate Acetate (PMA). Results are express as in nanomoles of H_2O_2 per $2x10^5$ cells, by comparing OD with standard curve of known H_2O_2 concentration.

Nitrite production

NO_2^- production by the macrophages culture was assessed through colorimetric method based on Griess Reaction (13). NO production was analyzed by the addition or not of IFN-γ. Results are express as in micromoles (umoles) of NO per $2x10^5$ cells, by comparing OD with standard curve of known NO_2^- concentration.

Tumour necrosis factor (TNF-α) bioassay

The murine tumor-line fibroblasts named L929, which is sensitive to TNF-α was used in the bioassay to detect this cytokine in serum and homogenates of splenic and hepatic cells from both lines through the procedure adapted from Di Giovine *et al* (9) and Pourshafie *et al* (26). TNF-α levels in the macrophages culture supernatants were calculated on a

standard curve plotted with recombinant TNF-α murine (R & D Systems, Minneapolis, MN, USA) used at concentrations from 40 to 4000 U / mL.

Statistical Analysis

To compare the serological titers obtained from mice H_{IV-A} and L_{IV-A} mice, parametric analysis of variance - ANOVA was used for independent samples, besides non-parametric Kruskal-Wallis test. Means of H_2O_2, NO and cytokine productions were compared by using repeated measures ANOVA and Student-Newman-Keuls t test for múltiple comparisons (29). Significance level was 5% for all tests.

RESULTS

The results expressed in Figures 1A and 1B indicate that the bacterial recovery from liver and kidneys occurred from the 4th day post-inoculation in both lines, peaking at the 7th day post-inoculation in H_{IV-A} and at the 7th, 14th and 21st days of infection in L_{IV-A} mice. After the 21st day, the values decreased. In L_{IV-A} line, the agent could be isolated for a longer period compared to $H_{IV-A.}$

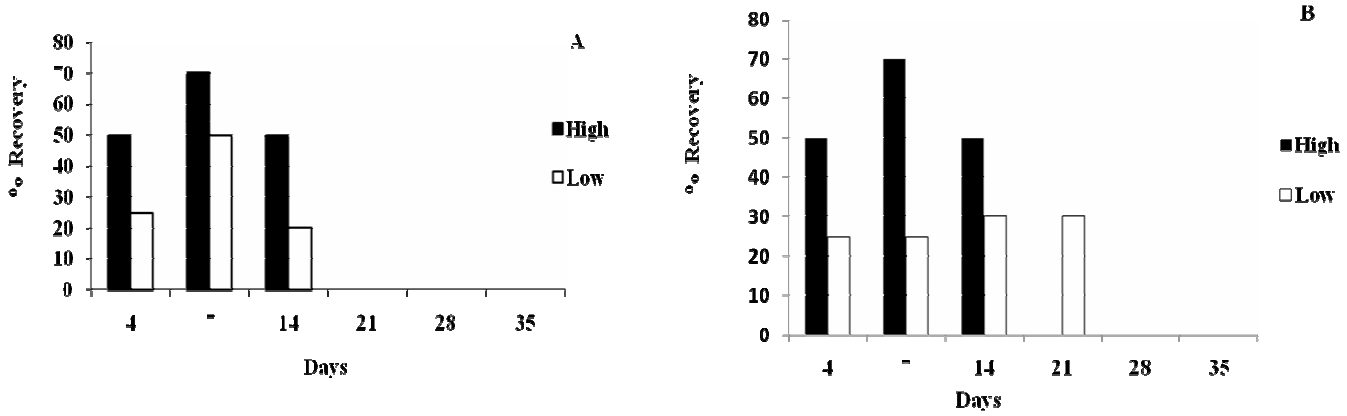


Figure 1. Recovery of leptospires from kidneys (A) and liver (B) of High and Low mice, which were inoculated with $2x10^7$ *L. interrogans* serovar Pomona in specific periods of time. Results are expressed as percentage of recovery.

In the present study, antibody production in the lines was associated with the leptospire recovery rates in renal and hepatic tissues. The antibody titers produced by mice are presented in Figure 2. In the negative control animals there was no detection of any titration values.

The analysis of such results indicates that there was no significant statistical difference ($p< 0.05$) between the genetically selected lines H_{IV-A} and L_{IV-A}, although antibody levels were higher in H_{IV-A} than in L_{IV-A}, especially between the 7th and 21st days post-inoculation.

The susceptibility and/or resistance were measured according the rate recovery of leptospires in culture, the presence of antibodies in a time graphical representation and by the characteristics of the lesions in kidney tissues through histopatological analysis (data not shown). Meanwhile, although the lines have different levels of production of antibodies and different rates of recovery of leptospires, mainly in the initial period of post-inoculation, they maintained the characteristics of resistance of the infectious agent has in the murine model.

As regards H_2O_2 production by peritoneal macrophages without stimuli, significant values were only observed at the

14th day in H_{IV-A} compared to L_{IV-A} lines (Figure 3A). When stimulated with IFN-γ, macrophages of control H_{IV-A} produced higher H_2O_2 concentration than those of control L_{IV-A} (Figure 3C). However, this apparent increase was not significant, probably due to the great variability of individual data.

The treatment of peritoneal macrophages with PMA significantly increased H_2O_2 production (Figure 3B). Comparing the production of H_2O_2 between lines, significant differences were observed at the 14th (H > L) and 21st (L > H) days of infection. The simultaneous stimulus with IFN-γ and PMA significantly increased H_2O_2 production at the 14th (H > L) and 21st (L > H) days of infection (Figure 3D).

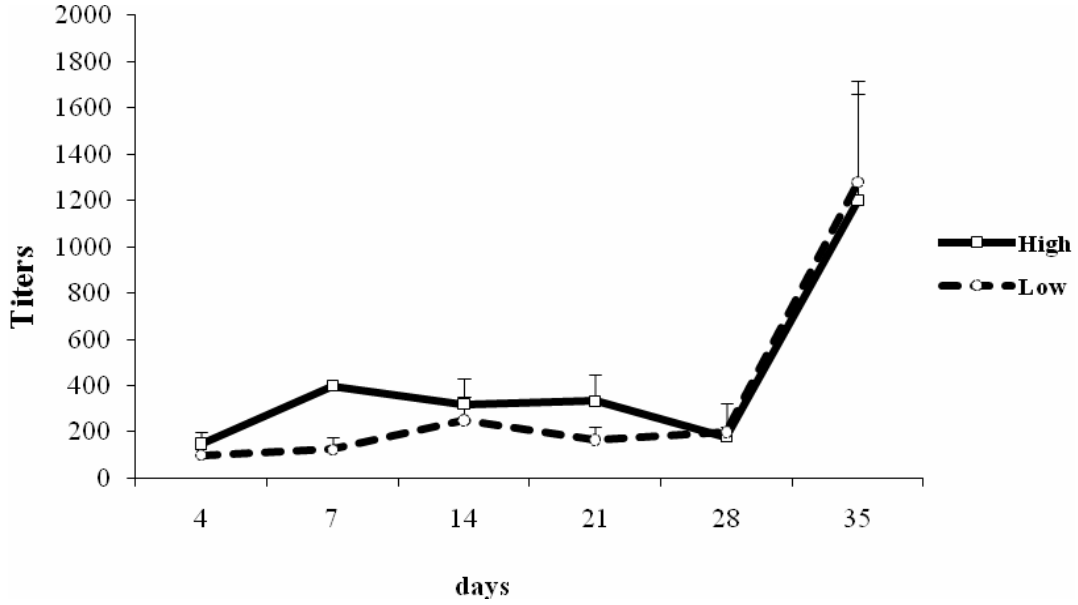
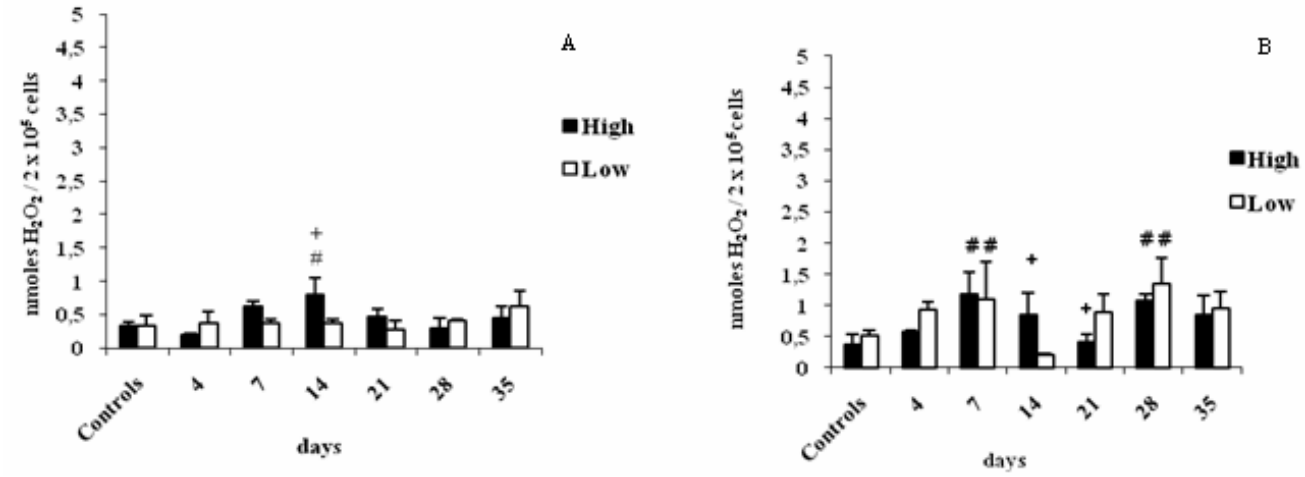


Figure 2. Antibody titers detected by microscopic agglutination test against the antigen *L. interrogans* serovar Pomona in High and Low mice inoculated with $2x10^7$ leptospires. Non-parametric Kruskal-Wallis test for independent samples (α = 0.05).



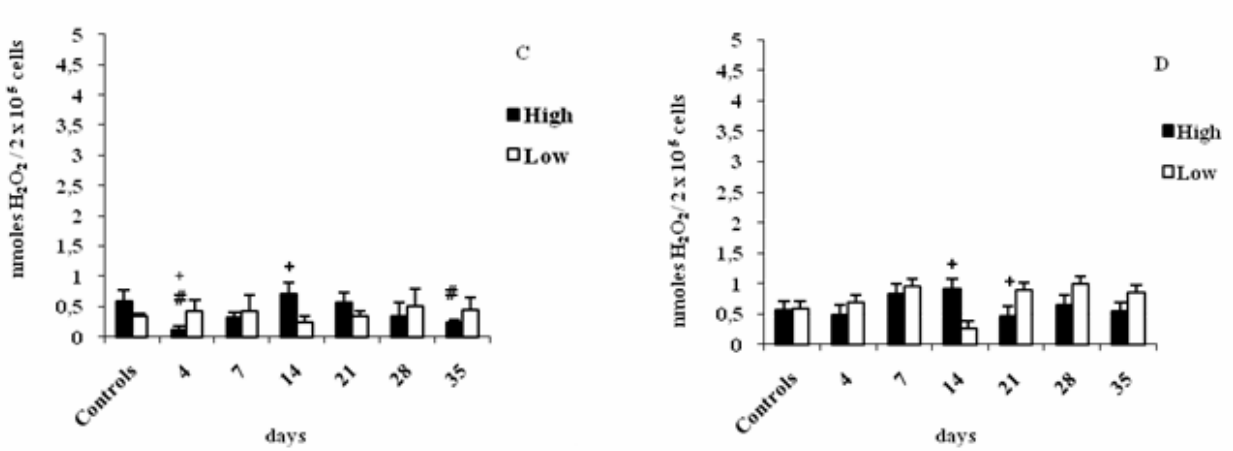
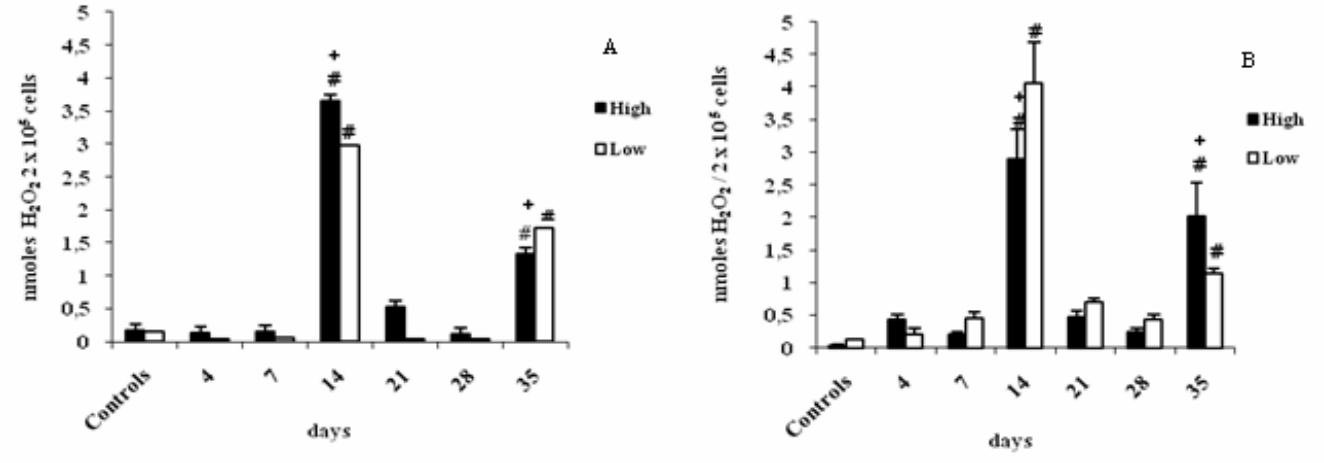


Figure 3. Hydrogen peroxide production by peritoneal macrophages of High and Low mice from the IV-A selection infected with *L. interrogans* serovar Pomona and not infected (controls). Macrophages were cultured in the absence of interferon-gamma (IFN-γ) without stimulus (A) or with PMA stimulus (B), or in the presence of IFN-γ without stimulus (C) or with PMA stimulus (D). Results represent the mean ± standard deviation of five animals per group. (#) indicates significant difference between infected and control mice, (+) indicates significant difference between lines (H x L). Student-Newman-Keuls test for multiple comparisons ($p < 0.05$).

H_2O_2 production by splenic macrophages without stimuli was higher at the 14th and 35th days in both H_{IV-A} and L_{IV-A} lines (Figure 4A). However, the treatment with IFN-γ resulted in differences between lines: H > L at the 21st day and L > H at the 35th day (Figure 4C). In PMA-stimulated macrophages, great alterations were observed at the 14th and 35th days between the infected lines and their respective controls, as well as between lines (Figure 4B). With the additional stimulus of IFN-γ and PMA, there was a difference between lines: L>H at the 14th day (Figure 4D).

As regards NO production by peritoneal macrophages, differences between lines were only detected at the 21st day, when L_{IV-A} produced more NO than H_{IV-A} mice (Figure 5A). The treatment with IFN-γ increased NO production in both lines over the experiment (Figure 5B). NO production by splenic macrophages stimulated or not with IFN-γ was not significantly different between infected mice and their respective controls, as well as between H_{IV-A} and L_{IV-A} lines.



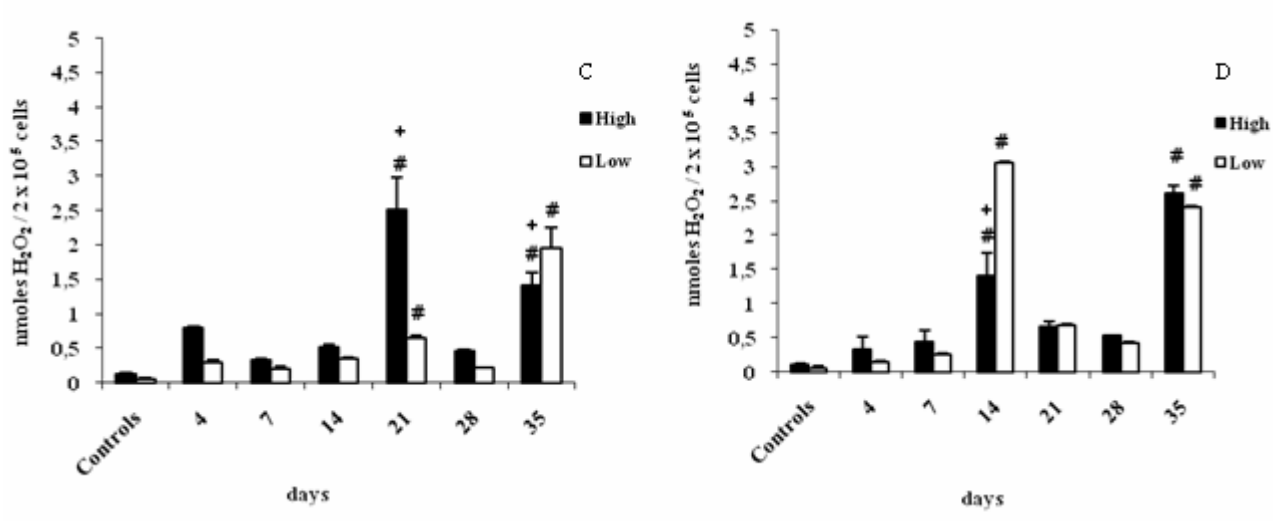


Figure 4. Hydrogen peroxide production by splenic macrophages of High and Low mice from the IV-A selection infected with *L. interrogans* serovar Pomona and not infected (controls). Macrophages were cultured in the absence of interferon-gamma (IFN-γ) without stimulus (A) or with PMA stimulus (B), or in the presence of IFN-γ without stimulus (C) or with PMA stimulus (D). Results represent the mean ± standard deviation of five animals per group. (#) indicates significant difference between infected and control mice, (+) indicates significant difference between lines (H x L). Student-Newman-Keuls test for multiple comparisons ($p < 0.05$).

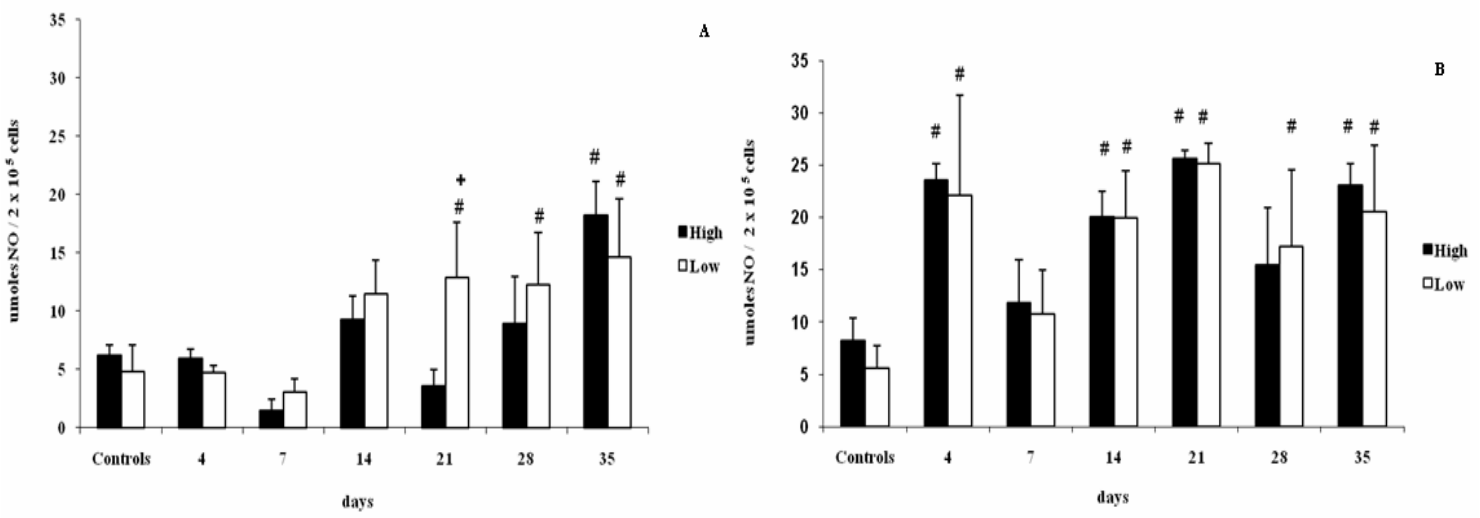


Figure 5. Nitric oxide production by peritoneal macrophages of High and Low mice from the IV-A selection infected with *L. interrogans* serovar Pomona and not infected (controls). Macrophages were cultured without stimulus (A) or with interferon-gamma stimulus (B). Results represent the mean ± standard deviation of five animals per group. (#) indicates significant difference between infected and control mice, (+) indicates significant difference between liness (H x L). Student-Newman-Keuls test for multiple comparisons ($p < 0.05$).

TNF-α production in serum and splenic cells of control H_{IV-A} and L_{IVA} mice was not significantly different. However, differences between lines were observed in the serum of L_{IV-A} line, which produced more TNF-α than H_{IV-A} at the 21th day post-inoculation (Figure 6A). In splenic cells, significant differences were observed at the 4th and 35th days of infection, when H_{IV-A} line produced more TNF-α than L_{IV-A} (Figure 6B). TNF-α production by hepatic cells was higher than that observed in other analyzed tissues and fluids. In the liver, H_{IV-A} mice had a significant inhibition relative to controls at the 7th and 14th days, recovering at the subsequent periods with a high production at the 21st, 28th and 35th days (Figure 6C).

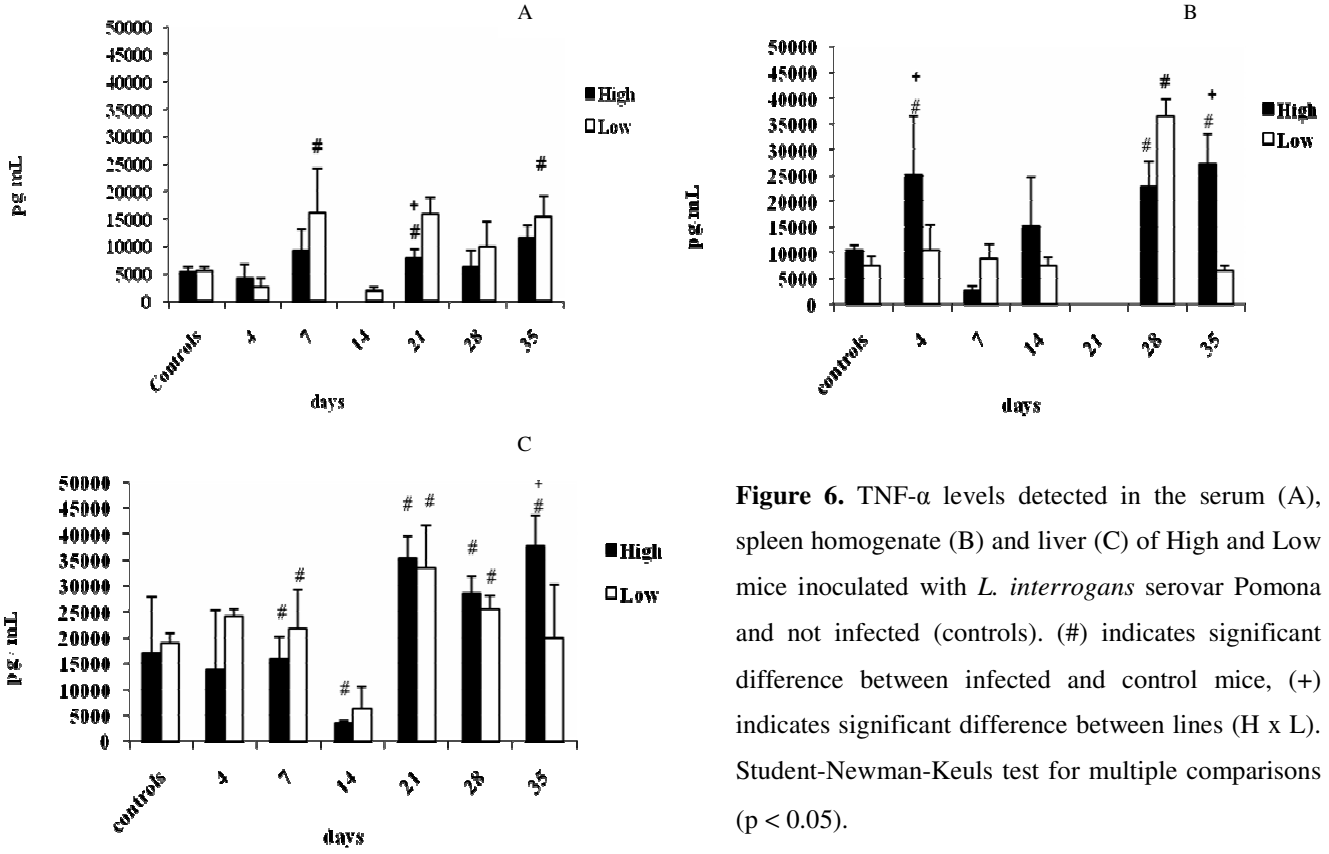


Figure 6. TNF-α levels detected in the serum (A), spleen homogenate (B) and liver (C) of High and Low mice inoculated with *L. interrogans* serovar Pomona and not infected (controls). (#) indicates significant difference between infected and control mice, (+) indicates significant difference between lines (H x L). Student-Newman-Keuls test for multiple comparisons ($p < 0.05$).

DISCUSSION

Mouse lines selected for the differentiation in the humoral response may present varied degrees of antigenic response due to genetic variations in immunoglobulin production and macrophagic activity.

In the present study, the infectious agent was recovered until the 21th day from the liver of L_{IV-A} mice, whereas H_{IV-A} mice completely controlled the infectious process in the same period. Such results may be related to the higher metabolism of macrophages from low-responder relative to high-responder animals, making difficult for macrophages from L_{IV-A} mice to present antigenic determinants to specific antibodies.

However, the formation of circulating antibodies probably reduced drastically the agent possibility of diffusion in tissues, contributing to the agent elimination after the 21th day in both lines, which made difficult the isolation of leptospires in later post-inoculation periods; this was also observed by Adler &

Faine (3, 4). The decreasing values of recovery from kidneys and liver coincide with periods of increased detectable immunoglobulin production in the microscopic agglutination test, demonstrating a positive correlation between these parameters, which corroborates other studies (22).

H_{IV-A} mice are more resistant than L_{IV-A} mice when the humoral immune response represents the main mechanism of protection against microorganisms. However, there was no significant difference in the production of antibodies against *L.* Pomona between H_{IV-A} and L_{IV-A} lines, although H_{IV-A} animals produced more antibodies between 7 and 21 days. These results partially disagree with those obtained by Marinho *et al* (22), who analyzed the production of antibodies against *L.* Icterohaemorrhagiae. H_{IV-A} line produced a statistically significant response relative to L_{IV-A}, maintaining the multispecific effect normally observed in such lines.

The endogenous production of hydrogen peroxide (H_2O_2) in peritoneal and splenic cells from H_{IV-A} line may have helped to control the infectious process, since a higher production of such metabolite was observed at statistically significant concentrations at the 14th day, which agrees with the results obtained in other studies (21). Although L_{IV-A} line presented a basal H_2O_2 production in most of the evaluated periods, this production was higher relative to controls at the 14th and 35th days in the spleen and at the 28th and 35th days in the peritoneum, when this line again completely controlled the infectious process.

As regards NO production, there were differences between lines considering infected animals without stimulus: L_{IV-A} produced more NO than H_{IV-A} in the peritoneum at the 14th, 21st and 28th days; in H_{IV-A} line this difference was only observed at the 35th day ($H_{IV-A} > L_{IV-A}$).

Comparing such results with those obtained for bacterial recovery, NO production contributed to control the infectious process at later infection periods (from the 14th day), which was similar to the results obtained by Marangoni *et al* (19).

Marinho (21) analyzed NO production in IV-A selection lines against the infection triggered by *Leptospira* serovar Icterohaemorrhagiae and observed that IFN-γ-treated cells had high NO levels. This effect was also observed in the present study. The treatment with IFN-γ and/or PMA on H_2O_2 production and with IFN-γ on NO production indicated that in some evaluated compartments and periods *L.* Pomona was a "messenger" for this cell to produce such metabolite.

Darrah *et al* (7) studied knockout mice to evaluate H_2O_2 and NO production and observed that the combination of both radicals resulted in a synergistic effect, with rapid and efficient pathogen killing by activated macrophages, especially under IFN-γ stimulus. In the present study, a positive interaction between both metabolites seems to have occurred, since such intermediaries were higher, especially in the infectious process control periods.

NO production requires the presence of IFN-γ, which plays an important role as a macrophage activating factor and a primary signal for the transcription of the enzyme Inducible Nitric Oxide Synthase (INOS). In innate immunity, signals for macrophage activation may originate from the pathogen and natural killer cells (NK cells), which are a source of IFN-γ (1).

In addition, Marinho *et al* (22) analyzed TNF-α production by H_{IV-A} and L_{IV-A} lines against *Leptospira* serovar Icterohaemorrhagiae and observed a high production in L_{IV-A} at the beginning of the infection and this level remained stable until the 14th day. Following this period, there was an inhibition of TNF-α production, exactly at the moment leptospires were not recovered anymore.

Production of tumor necrosis factor (TNF-α) as a parameter for macrophagic activity was also evaluated in the present experiment. TNF-α levels in the serum from infected L_{IV-A} line were higher at the 7th, 21st and 35th days. However, significant differences between lines were only observed at the 21st day. H_{IV-A} splenic cells produced more TNF-α than those of L_{IV-A} at the 4th and 35th days post-inoculation. In hepatic cells, TNF-α production was different between lines only at the 35th day ($H_{IV-A} > L_{IV-A}$).

These results indicate that TNF-α was an adjuvant in the

infectious process control in both lines, and the analysis of the production of this cytokine in H_{IV-A} hepatic cells evidenced its importance in the infection control, which agrees with the data obtained by Marangoni *et al* (20), who analyzed TNF-α production by hepatic macrophages, correlating it to the presence of lipopolysaccharides (LPS) from *Leptospira* serovar Icterohaemorrhagiae.

The analyses of other cytokines can be determinant to evaluate the profile of a broaden immune response of the different serovars of leptospires in the lines genetically selected. By Marinho *et al*. (22) the line H_{IV} had the profile Th2 with more production of antibodies, IL-4 and worst tissues lesions, while the line L_{IV-A} had the profile Th1 with higher production of IFN-γ, higher macrophage activity and lesser damaged tissues.

The differences in the recovery rates and in the cellular and humoral immune response of *Leptospira* serovar Pomona relative to other serovars investigated in previous studies may be related to antibody production control mechanisms and differentiated cellular response, but especially to the infectious agent adaptability factors, since serovars had different virulence in some hosts, i.e. they are species-adapted (14, 17).

In general, the study of the infection by *Leptospira* serovar Pomona in lines indicated that macrophagic activity and TNF-α production play an important role in the infectious process control, which varied according to the evaluated compartments and periods. Compared to studies with other serovars, the present results indicate partial differences in an immune trait genetically selected by an external nonspecific agent.

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