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Original article

The effect of LPS injections on non-specific immune response in affected pigeons

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Abstract

The aim of the study was to investigate the effect of LPS injections on non-specific mechanisms of immunity in pigeons. On the first day of observation the experimental birds (n=18) were intravenously injected with *Escherichia coli* LPS (10 μg/kg b.w.), while the control animals (n=6) received in the same way apyrogenic physiological saline. On the second and the third day of the experiment LPS in the same doses was injected again. Four and a half hours after the saline and each pyrogen administration blood samples were collected from the control and experimental pigeons. The total protein, gamma globulin, lysozyme, acute phase protein (Cp, CRP, Tf, ferritin, Alb) and selected trace element (Fe, Cu, Zn) concentrations were investigated. The obtained results showed the increase in the concentration of total protein, Cp, CRP and Tf in endotoxin fever resulting from LPS injection in pigeons. In contrast, the concentration of gamma globulins, ferritin and Alb were decreased in response to the first LPS injection. However, the consecutive injections of LPS caused a decrease in the concentration of total protein, CRP and Tf. In opposition to those results, a significant rise in the lysozyme and ferritin concentrations was observed. On the other hand, the first LPS injection caused a decline in the iron and zinc concentrations which remaining lower than the control values following repeated administration of LPS. On the contrary, the copper concentration increased successively in response to the next LPS injections.

Key words: pigeons, lipopolysaccharide, non-specific immune indices

Introduction

Lipopolysaccharide (LPS, endotoxin) belongs to the most often applied bacterial pyrogen in the studies on mechanism of fever and pyrogenic tolerance in birds (Johnson et al. 1993, Maloney and Gray 1998, Koutsos and Klasing 2001). It is known that LPS is responsible for many immunological changes in birds such as stimulation of IL-1β (Koutsos and Klasing 2001, Leshchinsky and Klasing 2001), MGF, INF-γ or TGF-β

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(Leshchinsky and Klasing 2001), cellular immune response as a result of changes in leukocyte subsets (Dudek and Bednarek 2011), lysozyme transformations (Bruckmaier 2005) as well as acute phase response (APR) manifesting as an intensified synthesis of acute phase proteins (APPs) (Johnson et al. 1993, Koncicki 2003). APP production is mediated by some cytokines, such as IL-1 (Klasing et al. 1987), IL-6 (Amrani et al. 1986) and TNF-α (Koh et al. 1996) and under a control of glycocorticoids (Amrani et al. 1986) and microelements such as iron, zinc and copper (Klasing and Johnstone 1991) constituting compounds of many enzymes taking a part in this process. Moreover, the mentioned trace elements are very important for bacteria proliferation during the infection. Therefore, similarly to mammalian organisms, birds developed a natural mechanism classified as a nutritional immunity (Mazur-Gonkowska et al. 2002). This status inhibits the bacterial mitotic division and proliferation due to the lack of agents stimulating their multiplication. In birds, two criteria of APP identity exist. The first is based on changes in the APP concentrations during APR. There are proteins which concentration increases (positive proteins), such as ceruloplasmin (Cp) or transferrin (Tf) (Mazur-Gonkowska and Koncicki 2002, Mazur-Gonkowska et al. 2003, 2004) and proteins which concentration decreases (negative, e.g. albumin) (Mazur-Gonkowska et al. 2003). The second criterion is based on the kinetics of changes in the APP concentration in response to the impulse. There are proteins of the first and second order. The concentration of proteins of the first group increases rapidly, such as serum amyloid A. In contrast, a slower increase in the concentration is characteristic for proteins of the second order, such as Cp or Tf (Koncicki 2003). However, these data do not concern pigeons, in which changes of APP concentrations were not examined until now.

Generally, there are well known basic directions of immune response in a consequence of LPS administration in most species of birds, but till now these changes are still weakly understood in pigeons. Therefore, the aim of the study was to investigate the effect of LPS injections on selected non-specific immune indices in this species of birds.

Materials and Methods

Animals

The study was performed on pigeons (n=24) aged 1-2 years with average body weight of 246-416 g, maintained in a stable climatic room (room temperature 21±1°C, relative air humidity 60%), and natural day/night cycle. The birds were kept in wooden cages (6 pigeons per cage) and fed with a standard fodder recommended for pigeons, with water *ad libitum*. The experiment was approved by the Local Ethic Committee on Animal Experimentation of the Agricultural University of Lublin, Poland.

A condition of endotoxin fever and pyrogenic tolerance was obtained as described by Dudek et al. (2011).

Injections:

Pigeons were divided into two groups: experimental (n=18) and control (n=6). On the first day of the study a state of endotoxin fever was evoked in the experimental birds. The experimental animals (group I) received *Escherichia coli* LPS (Serotype O111:B4, Sigma) in a dose of 10 μg/kg b.w. (10 μg LPS suspended in 1 ml saline) in the form of one intravenous injection, whereas the control pigeons (group II) were injected with apyrogenic saline at the dose of 1 ml saline/kg b.w. In both cases the final volume of the two solutions used was comparable and dependent on the body weight of individual pigeons.

Four and a half hours after the first LPS or saline injection, blood samples were collected from the control pigeons and from six randomly selected birds from the experimental group, labeled as LPS1.

On the following, the second and the third day of the experiment a pyrogenic tolerance was induced in the rest of the experimental pigeons. In order to induce pyrogenic tolerance twelve experimental birds were injected intravenously with *E. coli* LPS (Serotype O111:B4, Sigma) in a dose of 10 μg/kg b.w. (10 μg LPS in 1 ml saline) on the second day of the experiment. Four and a half hours after the second LPS injection blood samples were collected from the next six birds randomly selected from the remaining experimental animals, labeled as group LPS2. On the third day of the experiment the last six experimental pigeons received intravenously a third dose of *E. coli* LPS (10 μg LPS in 1 ml saline) and after 4.5 h blood samples were collected from the birds, which were labeled as group LPS3.

LPS or apyrogenic saline was intravenously injected into the ulnar vein (*vena ulnaris*) and blood samples for laboratory investigations were collected from the same vein at 24 h intervals in pigeons recruited from the LPS1, 2 and 3 subgroups.

The assays

Total protein concentration (Tp)

The concentration of Tp (g/L) was determined using commercial kit based on the biuretic method (Sigma).

Total gamma globulin concentration

To determine the gamma globulin concentration (g/L) the spectrophotometric method was applied as described by Siwicki and Anderson (Siwicki and Anderson 1993a).

Lysozyme activity

The lysozyme activity was measured by the turbidimetric method as described by Hansen (Hansen 1974) in Siwicki's modification (Siwicki and Anderson 1993b).

Ceruloplasmin concentration (Cp)

The concentration of Cp (IU) was determined using the spectrophotometric method as described by Rice et al. (Rice et al. 1986).

C-reactive protein (CRP), ferritin and transferrin (Tf) concentrations

CRP, ferritin and Tf alternations were measured using commercial kits manufactured by BioSystems according to the turbidimetric method described previously (Kreutzer 1976, Otsuji et al. 1982, Bernard and Lauwerys 1984).

Albumin concentration (Alb)

The Alb concentration was determined using the commercial kit (BioSystems) based on the spectrophotometric method described previously by Doumas et al. (Doumas et al. 1971).

Iron, copper and zinc contents

A determination of iron, copper and zinc concentrations in the blood serum of pigeons was performed using the X-ray fluorescence spectrometer (Oxford 2000; Oxford Instruments, Buckinghamshire, UK). This method is based on performing the measurement of the x-ray spectres characteristic for individual elements. The concentration of elements in the μg/g (ppm) was obtained by applying the H4 and H8 external standards (Atomic Energy Agency, Vienna, Austria). This method is based on calculating the surface area under the peak corresponding to the K line of the characteristic x-rays of given element which, after the standardization, was converted to concentrations with using calibration factors calculated from standard spectres. In the determination of the concentration of iron, zinc and copper in the blood serum of pigeons an alternative setting of the apparatus was used (anodic voltage of 35 kV, current 0.2 mA), allowing to reach the optimized level of the signal to noise ratio in recorded spectrums (Wróbel et al. 2004).

Data analysis

Results were presented as arithmetic means with standard errors (means ± SEM) after their statistical analysis with the Stat View 512 (Abacus Concepts, Berkeley, CA, USA) or STATISTICA 6.0 software. In order to compare several groups against each other Tukey's analysis of variance (for different N), Fisher's LSD or Dunnett's test were used. $P<0.05$ was taken as the statistical significance threshold.

Results

Tp concentration

The first LPS injection caused an increase in the Tp concentration as compared with the controls. In response to the second administration of pyrogen a decrease in this concentration was observed. A decline of the Tp value was deepened following the third LPS injection (Table 1).

Gamma globulin concentration

A decrease in the gamma globulin concentration was deepened following subsequent injections of LPS. Significant differences in the gamma globulin concentration between the examined groups are presented in Table 1.

Lysozyme activity

The first injection of LPS caused an increase in the lysozyme activity as compared with the control. This rise was augmented following the subsequent LPS injections. There were observed significant differences between LPS3 and control group (Table 1).

Cp activity

In response to the first LPS injection a rise in the Cp activity was registered. This rise was augmented following the third injection of pyrogen. In contrast, the second LPS injection caused a decline in the Cp activity which was lower than the control value (Table 1).

Table 1. The mean values of selected non-specific immune parameters in response to LPS injections in pigeons (mean ± SEM).

Parameter	Group			
	Control	LPS1	LPS2	LPS3
Total protein (g/L)	46.32 ± 2.27	51.63 ± 2.08	48.65 ± 2.96	46.90 ± 2.03
Gamma globulins (g/L)	17.22 ± 2.93	14.61 ± 2.27	5.92 ± 1.29[ab]	9.33 ± 1.11[a]
Lysozyme (mg/L)	13.61 ± 1.43	14.16 ± 1.11	14.66 ± 0.92	18.10 ± 2.03[a]
Acute phase proteins				
Cp (IU)	59.05 ± 1.63	60.18 ± 1.11	57.33 ± 1.28	61.11 ± 1.77
CRP (mg/L)	53.38 ± 3.00	53.46 ± 2.57	53.33 ± 3.18	53.06 ± 1.56
Tf (mg/dL)	16.00 ± 1.41	19.5 ± 2.49	18.66 ± 7.21	10.00 ± 1.37
Ferritin (μg/L)	54.02 ± 0.99	21.60 ± 0.84[ac]	151.26 ± 0.69[a]	97.24 ± 0.53[abc]
Alb (g/L)	12.61 ± 0.60	10.92 ± 0.39[a]	11.85 ± 0.57	12.70 ± 0.72[b]
Microelements				
Iron (μg/g)	157.48 ± 16.71	156.85 ± 26.02	82.13 ± 13.11[ab]	91.20 ± 5.95[ab]
Zinc (μg/g)	27.36 ± 1.42	23.25 ± 2.00	24.40 ± 1.73	24.08 +0.59
Copper (μg/g)	8.21 ± 0.36	8.69 ± 0.81	8.87 ± 0.39	10.31 ± 0.77[a]

LPS1 – the first LPS injection; LPS2 – the second LPS injection; LPS3 – the third LPS injection;
[a] – statistically significant differences at P<0.05 in comparison with the control
[b] – statistically significant differences at P<0.05 in comparison with LPS1
[c] – statistically significant differences at P<0.05 in comparison with LPS2

CRP concentration

The first injection of LPS caused a slight increase in the CRP concentration as compared with the control. In contrast, in response to the second administration of pyrogen the CRP decline was observed below the control values and it was deepened following the third injection of LPS (Table 1).

Tf concentration

In response to the first LPS injection an increase in the Tf concentration was observed as compared with the control. The consecutive injections of pyrogen caused its decline down to 10.0 ± 1.37 mg/dL for group LPS3 (Table 1).

Ferritin concentration

The first injection of LPS caused a decrease in the ferritin concentration as compared with the control. In contrast, in response to the second administration of pyrogen a sudden increase in the ferritin concentration was observed. This increase was still present following the third injection of LPS. The differences between various groups are summarised in Table 1.

Alb concentration

The first and second injection of LPS caused a decrease in the Alb concentration as compared with the control. In contrast, the third injection of LPS caused a subsequent rise in the Alb concentration which exceeded control values (Table 1).

Iron, copper and zinc contents

The first injection of LPS caused a slight decrease in the iron concentration as compared with the control. However, this fall deepened clearly after the second administration of pyrogen and achieved significantly lower values (P<0.05) as compared with LPS1 and control groups. After the third LPS injection the iron concentration was still below the control and LPS1 values (Table 1).

In response to the first LPS injection the zinc concentration in the blood serum of pigeons was close to the control values. A decrease in the zinc concentration was observed following the second and third administration of pyrogen (Table 1).

In comparison with the controls the consecutive LPS injections caused a gradual increase in the copper concentration. Only the differences between LPS3 and control group were significant at P<0.05 (Table 1).

Discussion

Our previous results showed that the injection of LPS caused a significant increase of internal temperature and a distinct decrease of locomotor activity in pigeons, evoking a state of endotoxin fever. In these conditions, general leukopenia and an increase in percentage of peripheral blood lymphocytes (CD 3^+, CD 4^+ and CD 8^+) were also observed (Dudek 2007, Dudek and Bednarek 2011).

The results indicated an increase in the Tp concentration in blood sera of pigeons following the first LPS injection. This rise was a result of increased activity of lysozyme and some APPs, such as Cp, CRP, Tf and probably other serum proteins. On the other hand, a decline was observed in the ferritin and Alb concentrations. An increase in the CRP concentration observed in our study makes an evidence that this protein belongs to positive APPs in birds which concentration increases during APR (Klasing and Johnstone 1991). Additionally, in this study a rise in the Cp concentration was observed at four and a half hours after the injection of LPS. Other study showed that the increased concentration of ceruloplasmin in the plasma of chickens was still present 24 hours following the pyrogen administration (Baert et al. 2005). A significant stimulation of Cp production was also observed in condition of experimental infection with *E. coli* in turkeys. This stimulation was still evident 240 hours after the infection (Mazur-Gonkowska et al. 2003, 2004). The rise in the Cp concentration was probably related to an activation of mechanisms which protect the host organism from the destructive effect of APR. A protective function of this protein is associated with a transport of about 95% of copper plasma pool (Butler and Curtis 1977) which increased content was observed in our study in pigeon sera and in plasma of other birds following the LPS injection (Johnson et al. 1993). This α2 globulin (Butler and Curtis 1977) is responsible for cellular membrane stabilization (Koh et al. 1996), protection from excessive accumulation of activated oxygen radicals in phagocytic cells (Butler and Curtis 1977) and reversion a hypoferremia accompanying APR (Piercy 1979). In the last mechanism a significant role is played by iron-binding glycoprotein – Tf. This protein is able to reduce the iron content in both the serum and plasma which was observed in the conditions of endotoxin fever in our study in pigeons as well as in other birds (Johnson et al. 1993). Therefore, Tf plays an important pro-immunological role because of a limitation of bacterial proliferation as a consequence of iron deficiency (Zimber et al. 1985, Hallquist and Klasing 1994). Infectious hypoferremia was also found in other study following the experimental infection with *E. coli* in turkeys. This state was manifested with a decrease in levels of total iron and percent transferrin saturation and an increase in the total iron binding capacity and unsaturated iron binding capacity levels (Mazur-Gonkowska and Koncicki 2002, Mazur-Gonkowska et al. 2004). Other iron-binding protein is ferritin. Our results indicated a decrease in the ferritin concentration in the blood sera of pigeons following the first injection of LPS. This decline was probably associated with the iron binding and its transport in the host organism. Alb constitutes about two thirds of proteins of the blood serum and, among others, has transporting function for some inorganic and organic substances (Spano et al. 1988). Alb is negative APPs in the poultry and its concentration decreases during APR (Koncicki 2003). It was confirmed in our study where a decline in the Alb concentration following the first LPS injection in pigeons was observed. A decrease in Alb concentration was also observed in other study in the experimental infection with *E. coli* in turkeys (Mazur-Gonkowska et al. 2003, 2004).

Our results indicated a decrease in the zinc concentration in blood sera of pigeons at four and a half hours after the injection of LPS. Similar results were found in plasma of chickens (Johnson et al. 1993) and Japanese quails (Koutsos and Klasing 2001) at 16 hours after the pyrogen administration. A decrease in the zinc concentration in conditions of endotoxin fever was probably associated with an increase in the metalotionein concentration which is responsible for binding and transporting of zinc. A rise in this protein concentration was noted during APR in birds (Hallquist and Klasing 1994).

In our study a decrease in the total protein concentration following the second LPS injection in pigeons was observed in comparison with group LPS1. This decline was probably a result of significant decrease in gamma globulins and some APPs such as Cp, CRP and Tf content. A decrease in the Cp concentration probably affected a rise in the content of copper observed in this group with reference to the first injection of LPS. On the other hand, in response to the second LPS injection, an increase in the lysozyme and Alb concentrations and a significant rise in the ferritin content were observed as compared with group LPS1. The third LPS injection caused a consecutive decrease in the total protein concentration as compared with group LPS2. This decline was a result of decreased values of ferritin, CRP and Tf concentration. A decrease in the Tf concentration could correlate with a slight increase in the iron concentration in comparison with after the second pyrogen administration. Additionally, in response to the third LPS injection increased concentrations of gamma globulins, lysozyme and some APPs such as

Cp and Alb were found in comparison with group LPS2. In spite of the increased Cp concentration the third pyrogen injection caused consecutive rise in the copper content in comparison with double LPS injection. In response to the second pyrogen administration an increased concentrations of zinc was observed as compared with group LPS1. However, the third injection of LPS caused a slight zinc content decline in comparison with group LPS2. In contrast, other study showed a decrease in the zinc concentration in blood sera of Japanese quails following double LPS injection in comparison with the single pyrogen administration. On the other hand, the opposite results were observed in these birds following triple pyrogen injection in comparison with a double administration (Koutsos and Klasing 2001). These differences could be a result of different kind and dose of pyrogen used in this study which was LPS of *Salmonella* Typhimurium in a dose of 7.5 mg per kg b.m. On the other hand, in our study the time of blood collection was four and a half hours after each LPS injection, whilst in that study it was 11.5 hours later (Koutsos and Klasing 2001).

Our results show a decrease in the gamma globulin concentration in blood sera of pigeons following the first LPS injection. This fall could correlate with the phenomenon of the LPS opsonisation, due to a reduction in the content of free immunoglobulins of individual classes of gamma globulin fraction circulating in the blood. These immunoglobulins, components of complement and alien molecules coated with them, are binding the surface of phagocytes. A phagocytosis of these molecules by neutrophils occurs, among others, with the participation of factors present in cytoplasmic granules of these cells. One of them is an enzyme with antibacterial properties – lysozyme (muraminidase), which action is also associated with a nonenzymatic mechanisms (Gołąb et al. 2002). In the process of alien molecules destruction lysozyme is released from the granules of phagocytes what results in an increase of its concentration. This rise was observed in our study following the first LPS injection in pigeons (endotoxin fever) what is a proof of the activation of important humoral compound of non-specific immune mechanisms (Gołąb et al. 2002). Some cytokines are responsible for the stimulation of APP production in birds (Amrani et al. 1986, Klasing et al. 1987, Klasing and Johnstone 1991, Koh et al. 1996). A depression of the cytokine production observed in response to repeated LPS injections could affect a fall in APP concentrations in conditions of pyrogenic tolerance in pigeons. On the other hand, the increased lysozyme and gamma globulin contents may indicate the activation of non-specific immune mechanisms in these conditions.

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