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Original article

Cellular immune response of pigeons in the conditions of endotoxin fever and pyrogenic tolerance

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Abstract

The aim of this study was to investigate changes in selected parameters of cellular immune response in the conditions of endotoxin fever and pyrogenic tolerance in pigeons. On the first day of observation the experimental birds (n=18) were intravenously injected with *Escherichia coli* LPS at a dose of 10 µg/kg b.w., while the control animals (n=6) received apyrogenic physiological saline also in the form of injection. On the second and the third day of the experiment LPS was injected additionally at 24 h intervals. Four and a half hours after the saline and pyrogen administration blood samples were collected from the control and experimental pigeons. The following immunological assays were performed: WBC, leucogram and immunophenotyping of lymphocyte subsets in peripheral blood, i.e. CD 3⁺ (T lymphocytes), CD 4⁺ (T helper lymphocytes) and CD 8⁺ (T suppressor/cytotoxic lymphocytes) cells. In the conditions of endotoxin fever (i.e. after the first LPS injection) leucopenia, monocytopenia, heterophilia and eosinophilia were observed. Additionally, the immunophenotyping of peripheral blood lymphocytes indicated an increase in percentage of CD 3⁺, CD 4⁺ and CD 8⁺ cells in response to the single injection of LPS. In contrast, the consecutive injections of LPS, which created a pyrogenic tolerance effect, caused a decrease in WBC value, heteropenia, eosinopenia and lymphocytosis. Moreover, during this state an increase in percentage of CD 3⁺ and CD 8⁺ cells was demonstrated in contrast to the percentage of CD 4⁺ lymphocytes. The general tendencies in cellular immune response of the affected pigeons in the conditions of endotoxin fever and pyrogenic tolerance aim at activation of defence mechanisms against LPS for its prompt elimination from the animal's organism.

Key words: pigeons, endotoxin fever, pyrogenic tolerance, cellular immune response

Introduction

Fever is one of the basic reactions of organisms to infection, and is generally included in immunological response manifestations (Roberts 1991). Exogenous pyrogens, such as lipopolysaccharide (LPS) are involved in the induction of febrile rise of internal tem-

perature (Blatteis and Sehic 1997). LPS is isolated from different species of Gram-negative bacteria e.g. *Escherichia*, *Salmonella*, *Pseudomonas*, *Vibrio* (Henderson and Wilson 1996). LPS is effectively able to induce fever in different species of birds, i.e. chickens (Jones et al. 1983, Gregorut et al. 1992, Johnson et al. 1993, Macari et al. 1993), quails (Koutsos and Klasing

2001), ducks (Maloney and Gray 1998) and pigeons (Nomoto 1996, 1997), depending on pyrogen origin (Jones et al. 1983, Gregorut et al. 1992), and the time of administration of pyrogens (Nomoto 1996, 1997), as well as age (Jones et al. 1983, Gregorut et al. 1992, Koutsos and Klasing 2001), applied species of birds (Leshchinsky and Klasing 2001), dose (Johnson et al. 1993, Maloney and Gray 1998, Koutsos and Klasing 2001, Leshchinsky and Klasing 2001), and route (Johnson et al. 1993, Macari et al. 1993, Nomoto 1997). Apart from thermoregular changes LPS is also responsible for effects connected with behavior, e.g. depression of locomotor activity (Kozak et al. 1995), somnolence (Cady et al. 1989) or hypophagia and hypodipsia (Kozak et al. 1995). The important group of changes concerning action of LPS are metabolic alterations, such as: hypoglycaemia (Oguri et al. 2002) and influence on mineral balance of the organism (Johnson et al. 1993), as well as endocrinological changes, including activation of the hypothalamo-pituitary-adrenal axis (Gray and Maloney 1998, Kozak et al. 1998). Immunological changes constitute a separate group connected with stimulation of synthesis of some cytokines (Kozak et al. 1998), the effect on the leukocytic system (Krumrych et al. 1996), participation in lysozyme or laktoferin changes (Bruckmaier 2005), synthesis of antibodies (Mukezamfura et al. 1996), and a number of changes known as acute phase response (APR) (Johnson et al. 1993), during which intensification of synthesis of acute phase proteins (APPs) is observed (Baert et al. 2005). The changes accompanying APR are involved in the first line of defense mechanisms, activated during infection (Johnson et al. 1993).

Induction of pyrogenic tolerance is possible in response to bacterial pyrogens (Soszyński et al. 1991), e.g. LPS (Koutsos and Klasing 2001). Mechanisms of tolerance to LPS (endotoxin tolerance) are generally known, but its detailed aspects need further studies. Two phases of tolerance are distinguished. The first phase of endotoxin tolerance, known as early-phase pyrogenic tolerance, is characterized by the reduction of synthesis and release of proinflammatory cytokines as a consequence of the changes in the infected cell. Suppression of tumor necrosis factor synthesis (Matsuura et al. 1994) and inhibition of interleukin-6 (Roth et al. 1994) and nitric oxide (Chang et al. 1996) production belong to the main effects of the early-phase pyrogenic tolerance. Additionally, cellular immunity (Soszyński 2000) is stimulated, as a result of increased hepatic macrophage activation, responsible for LPS detoxication (Dinarello et al. 1968).

Very little is known about the immunological changes in response to LPS in birds, especially in the context of cellular immunity. So far there have been no data concerning this subject in pigeons. Therefore, the aim of the present study was to investigate the

effect of LPS on cellular immune response in pigeons in the conditions of endotoxin fever and pyrogenic tolerance.

Materials and Methods

Animals

The study was performed on pigeons (n=24) aged 1-2 years with average body weight 246-416 g, maintained in a stable climatic room (room temperature = $21\pm 1^\circ\text{C}$, air relative humidity = 60%), and natural day/night cycle. The birds were kept in wooden cages (6 pigeons per cage) and fed with 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.

Designation of endotoxin fever and pyrogenic tolerance state

The preliminary studies indicated that a single intravenous injection of LPS at a dose of 10 $\mu\text{g}/\text{kg}$ b.w. caused a statistically significant increase of internal temperature and distinct decrease of locomotor activity in pigeons, evoking a state of endotoxin fever. Four and a half hours after the first LPS injection the peak of internal temperature was observed. In contrast, the injection of pyrogen performed 3 times, at the same dose, caused a reduction of risen internal temperature, and an increase of locomotor activity of the birds in comparison to the single injection of LPS, showing that the 3 injections induce a state of pyrogenic tolerance (Dudek et al. 2010).

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) at a dose of 10 $\mu\text{g}/\text{kg}$ 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, second and third day of the experiment 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) at 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 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.

In order to determine the chosen cellular immune parameters the following assays were performed: total white blood cell count (WBC) with its differentiation (leucogram), and detailed peripheral lymphocyte immunophenotyping.

The total value of white blood cells (WBC) in peripheral blood of pigeons

To determine WBC the peripheral blood of pigeons was diluted in Natt-Harrick liquid in the ratio of 1 to 200. Leukocytes were counted in a Burker's chamber and the values were formulated in 10³/µl.

The percentage of leukocyte subpopulations in peripheral blood of pigeons (leucogram)

The blood smear was prepared, dried and stained using the Pappenheim method. The leukocytes, in a number not exceeding 100, were then counted with the use of an optic microscope. The values of the cells, i.e. granulocytes (heterophils, eosinophils and basophils) and agranulocytes such as lymphocytes and monocytes were given as a percentage (%).

Immunophenotyping of blood lymphocytes

Immunophenotyping of peripheral blood lymphocytes, i.e. CD 3⁺ (T lymphocytes), CD 4⁺ (T helper lymphocytes, Th), CD 8⁺ (T suppressor/cytotoxic lymphocytes, Ts/c) was determined using a panel of mice monoclonal antibodies (monoclonal antibodies, MCAs, Serotec), i.e. CD3 (CT-3 clone), CD4 (CT-4 clone)

and CD8 (CT-8 clone) conjugated with fluorescein-5-isothiocyanate (FITC). The chosen antibodies showed specificity for chicken lymphocytes, as indicated by the producer, however a cross-reactivity with pigeon lymphocytes has been confirmed experimentally (Dudek 2007). The determination of the above mentioned cluster of differentiation antigens (CD) on lymphocytes stained with appropriate antibodies was performed using a flow cytometer (Coulter Epics XL 4C, Beckam Coulter Company, USA). This determination was done by working procedure (Beckam Coulter Guide Procedure), whereas the SYSTEM II 3.0 and Multigraph program (Beckam Coulter) was used for archiving data from each acquisition in the form of "list mode", and for their analysis and presentation in the form of histograms.

Data analysis

Results were presented as arithmetic means with standard errors (means ± SEM) after their statistical analysis with the use of 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 performed. P<0.05 was taken as the statistical significance threshold.

Results

Total value of white blood cells (WBC) in peripheral blood of pigeons

In response to the first injection of LPS a decrease in the WBC count in the peripheral blood of pigeons was observed (Table 1). In contrast, the second injection of LPS caused a statistically significant (P<0.05) increase in the examined parameter as compared with group LPS1. A renewed fall in WBC total value was noted after the third administration of pyrogen. The differences between LPS3 and LPS2 groups of pigeons were statistically significant (P<0.05).

The percentage of leukocyte subpopulations in peripheral blood of pigeons

The first injection of LPS caused a statistically significant (P<0.05) increase in the percentage of heterophils in the peripheral blood of pigeons as compared with the controls (Table 1). The differences between group LPS2 and control was still significantly higher (P<0.05) in spite of a small decrease in heterophil values after the second administration of pyrogen.

Table 1. Mean values of selected parameters of cellular immune response in conditions of endotoxin fever and pyrogenic tolerance in pigeons ($\bar{x} \pm \text{SEM}$).

Parameter	Group			
	Control	LPS1	LPS2	LPS3
WBC ($10^3/\mu\text{l}$)	31.29 + 4.64	26.07 + 3.42	51.14 + 1.24 ^b	24.62 + 2.11 ^c
Leucogram				
Heterophils (%)	38.00 ± 1.46	65.00 ± 4.28 ^a	56.66 ± 9.67 ^a	20.83 ± 4.43 ^{abc}
Eosinophils (%)	0.16 ± 0.16	0.66 ± 0.42	0.16 + 0.16	–
Basophils (%)	–	–	–	–
Lymphocytes (%)	61.33 ± 1.52	34.16 ± 4.19 ^a	42.16 ± 9.26 ^a	79.00 ± 4.35 ^{abc}
Monocytes (%)	0.50 ± 0.22	0.16 ± 0.16	1.00 ± 0.44 ^b	0.16 ± 0.16 ^c
Phenotyping analysis of T lymphocytes				
T lymphocytes (CD 3 ⁺) [%]	27.54 ± 0.58	27.57 ± 1.22	27.60 ± 1.04	30.12 ± 1.20
Th lymphocytes (CD 4 ⁺) [%]	13.72 ± 0.74	16.00 ± 0.93	14.90 ± 1.27	12.90 ± 1.36 ^b
Ts/c lymphocytes (CD 8 ⁺) [%]	10.68 ± 0.83	14.75 ± 0.72 ^a	14.66 ± 0.75 ^a	15.76 ± 1.21 ^a

^a – statistically significant differences at $P < 0.05$ in comparison with 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

However, the value decreased further in group LPS3 and finally it was significantly lower in comparison with control, LPS1 and LPS2 groups of birds at $P < 0.05$.

The first administration of LPS caused a distinct increase in the percentage of eosinophils in the peripheral blood of pigeons as compared with the control and LPS2 group of birds (Table 1), in which the values were similar to each other. However, this rise was not statistically significant. In contrast, in response to the third injection of pyrogen no eosinophils in the peripheral blood of birds was observed.

In all experimental groups of pigeons and control no basophils were noted in the peripheral blood of birds (Table 1).

The first injection of LPS caused a statistically significant ($P < 0.05$) decrease in the percentage of lymphocytes in the peripheral blood of pigeons (Table 1), whereas in response to the consecutive injections of pyrogen a gradual increase in the examined parameter value was observed. The rise was statistically significant ($P < 0.05$) in LPS3 as compared with the control and last experimental groups of birds.

The second administration of LPS caused a statistically significant ($P < 0.05$) elevation in the percentage of monocytes in the peripheral blood of pigeons in comparison with the first administration of pyrogen (Table 1). The same value for this parameter was observed in both LPS1 and LPS3 groups of birds.

Immunophenotyping of peripheral blood lymphocytes in pigeons

Consecutive injections of LPS caused an increase in T lymphocytes (CD 3⁺) as compared with the controls (Table 1). The rise was small at the beginning of the study because the percentage of CD 3⁺ cells after the first and the second administration of pyrogen remained at a similar level to the controls. However, in response to the third injection of LPS a distinct increase in this parameter was observed. No statistically significant differences between examined groups of pigeons was noted.

A gradual decrease in the percentage of Th lymphocytes (CD 4⁺) was observed in response to consecutive administrations of LPS when compared to group LPS1 (Table 1). Statistically significant ($P < 0.05$) differences were observed only between LPS1 and LPS3 groups of pigeons.

The percentage of Ts/c lymphocytes (CD 8⁺) increased after the first injection of pyrogen and remained at a higher level until the end of the study as compared with the controls (Table 1). The percentage of CD 8⁺ cells after the second administration of LPS was similar to that observed in group LPS1. Statistically significant differences ($P < 0.05$) between experimental and control groups were noted in regard to this parameter.

Discussion

In conditions of endotoxin fever in the affected pigeons leucopenia was observed as a result of a significant ($P < 0.05$) lymphopenia, monocytopenia and a lack of basophils in the peripheral blood. In contrast, the single injection of LPS caused considerable ($p < 0.05$) heterophilia and an increase in eosinophil percentage. A distinct rise in the percentage of heterophils in the peripheral blood probably resulted from the production of these cells in bone marrow, accelerated migration of heterophils from marrow to peripheral blood, or from the marginal pool of blood vessels (Sitarska et al. 2000). In contrast to mammals, heterophils in birds derive from stem cells in the extravascular spaces of bone marrow (Campbell 1967), although this area is predominantly adapted for erythroid cell production and maturation in some birds (Glick and Rosse 1976). The cells probably descend from extramedullary sources too (Lawn 1979). In some birds, heterophilia appears as a control indicator of an interaction between bone marrow and the hypothalamic-pituitary adrenal cortical axis during different pathological states, such as hypoxia (Vanhooser et al. 1995) or riboflavin deficiency (Goff et al. 1953). Heterophils play an important role in phagocytosis, using factors included in cytoplasmic granulations (Macrae and Spitznagel 1975), such as hydrolytic enzymes (Maxwell and Robertson 1998), i.e. phosphorylase, acid phosphatase (Nair 1973), β -glucuronidase (Nair 1973, Rausch and Moore 1975), alkaline phosphatase or lysozyme (Rausch and Moore 1975). However, heterophil granulations are peroxidase-deficient in contrast to mammalian counterparts (Bainton and Farquhar 1968, Egami and Sasso 1991). It is worth recording that these cells probably take part in the first-line host defence (Ziprin 1997) and seem to be "efficient killers" with reference to *Escherichia coli* and other bacterial infections in chickens (Maxwell and Robertson 1998) and turkeys. The predominant immunological role of heterophils was seen after their opsonisation, when their phagocytic activity was noted to be enhanced in response to different species of bacteria (Harmon et al. 1992, Andreasen et al. 1996). Eosinophils also take part in the process of foreign cell destruction, and their increased percentage was observed in response to a single injection of LPS. The cells appear as "early natural modulators of inflammation" in birds (Maxwell 1987). However, their participation in phagocytosis is less important in comparison with neutrophils, considering the lack of lysozyme and phagocytin in heterophil granulations. In contrast a decrease in the percentage of monocytes in the pigeons after the single injection of LPS was observed, which probably resulted from cell migration into the site of infection, where the monocyte transformation to macrophages

(responsible for phagocytosis) is present (Traczyk and Trzebski 2001). In response to LPS a similar activation of macrophages and neutrophils is noted. Usually the activated cells stimulate the release of proinflammatory cytokines, i.e. IL- 1α and β , IL-6, TNF- α or IFN- α_2 (Toth and Blatteis 1996), known as mediators of fever response. Additionally, the cells are able to generate oxygen radicals following the activation of membrane-bound NADPH oxidase in order to eliminate the pathological microorganism (Rossi 1986, Cadenas 1989). In response to LPS the enhanced production of oxygen reactive forms, i.e. superoxide radical or hydrogen peroxide, by avian polymorphonuclear leucocytes is also present, similarly to mammalian counterparts (Dri et al. 1978). Therefore both heterophils and macrophages are probably involved in the first-line host defence (Cadenas 1989, Ziprin 1997). A significant ($P < 0.05$) lymphopenia was observed in the conditions of endotoxin fever in the pigeons. This probably resulted from the recirculation of lymphocytes from blood to regions dependent on thymus and marrow in peripheral lymphoid organs (Traczyk and Trzebski 2001). The immunophenotyping of peripheral blood lymphocytes in the pigeons demonstrated a small increase in the percentage of CD 3⁺ cells in response to the single injection of LPS. This was mainly a result of an elevated percentage of both CD 4⁺ and CD 8⁺ cells in these conditions. The rise in percentage of Th lymphocytes (CD 4⁺) indicated the mobilisation of immunological processes, i.e. the stimulation of proliferation and differentiation of B lymphocytes, and production of immunoglobins as a consequence of this state, whereas the increased Ts/c lymphocyte percentage suggests the activation of the ability to destroy and eliminate damaged or infected cells by means of antibody dependent cytotoxicity (Traczyk and Trzebski 2001, Gołab et al. 2002). It is worth mentioning that two subpopulations of Th lymphocytes, i.e. Th1 and Th2 are discerned. Th1 lymphocytes are mainly responsible for the mechanisms of cellular immunity, whereas Th2 only for humoral immunity. The first subpopulation possesses the ability to synthesize interleukin-2 and interferon- γ , and in this manner it intensifies the cytotoxicity of the lymphocytes and activates macrophages. Tc lymphocytes possess a secretory ability regarding cytokines similar to Th1 cells. The active mechanism of Tc lymphocytes mainly comes down to the cytotoxicity, which progresses through one of the two apoptotic pathways (Gołab et al. 2002). Therefore the increase in the percentage of Tc lymphocytes in conditions of experimental endotoxin fever may be proof of the direct participation of the cells in the control of endotoxemia. However, Ts lymphocytes are responsible for regulation of the functions of the remaining subpopulations of T lymphocytes (Traczyk and Trzebski 2001).

The state of pyrogenic tolerance after consecutive injections of LPS had a distinct effect on immunological response. Double administration of LPS caused a significant ($P < 0.05$) increase in WBC in peripheral blood in comparison with group LPS1. This was a result of a considerable ($P < 0.05$) increase in the percentage of monocytes and lymphocytes in the peripheral blood. Similarly, immunophenotyping of peripheral blood lymphocytes demonstrated a small rise of CD 3⁺ cells in group LPS2 as compared with the single injection of the pyrogen. This resulted from an increase in the percentage of other subpopulations of lymphocytes rather than Th and Ts/c cells, whose values decreased in these conditions. In contrast, heteropenia and eosinopenia were observed in this group of pigeons in comparison to the state observed after a single injection of LPS. This was probably a result of migration of heterophils and eosinophils from peripheral blood into tissues (Traczyk and Trzebski 2001). Additionally, a significant ($P < 0.05$) decrease in WBC in peripheral blood was observed in response to the third injection of the pyrogen in comparison with LPS2. This resulted from distinct ($P < 0.05$) heteropenia and monocytopenia and the lack of eosinophils in the blood as compared with the double administration of LPS. This probably indicates increased migration of most of the cells into tissues as a consequence of intense phagocytosis. In contrast, three injections of the pyrogen caused a significant ($P < 0.05$) rise in the percentage of lymphocytes in peripheral blood as compared with group LPS2. This was probably a result of the increased percentage of CD 3⁺ in the peripheral blood as a consequence of Ts/c cell rise. The increase in the percentage of Tc lymphocytes indicates the activation of cytotoxicity against infected cells (Traczyk and Trzebski 2001) as one of the components of immunological mechanisms (Gołąb et al. 2002).

The activation of components of APR in conditions of endotoxin fever in the pigeons, such as cellular immunity, indicates the defence of the organism against the damaging influence of LPS. Additionally, the reactivity of these immunological mechanisms accompanying pyrogenic tolerance in the birds led to increased elimination of the pathogenic factor from the pigeon organism.

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