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Thymic Cellular Immune Response from Females of the Migratory Gull *Larus ridibundus*

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Abstract: The immune response of thymic cells from female blackheaded gulls *Larus ridibundus* has been studied searching for changes associated to the migratory cycle. The functions assayed were those considered most representative of the cellular immune response: chemotaxis, lymphoproliferative response to PHA and Con A, and Natural Killer (NK)-like cell-mediated cytotoxicity. These functions were assayed in the post-migratory and the premigratory periods, since these migratory periods could produce the highest difference in the response. Experimental groups were constituted on the basis of the age of the animals, because this parameter is considered to induce differences in the immune response. Regarding the effect of the migratory cycle, it was obtained a different pattern of response depending on the function studied. Thus, lymphoproliferation and NK-like cell-mediated cytotoxicity did not show any migration-associated variation. Chemotaxis indexes were lower in the post-migratory period compared to the values obtained in the premigratory period in juvenile gulls. These findings demonstrate that the mobility of thymic cells is depleted after the long migratory flight in juveniles, suggesting that juveniles could be more sensitive to the stressful conditions of the migration.
Abbreviations: Con A, concanavalin A; cpm, counts per min; FBS, fetal bovine serum; FMLP, N-formyl-Met-Leu-Phe; NK, natural killer; PBS, phosphate-buffered saline solution; PHA, phytohaemagglutinin.

Key words: Gull, thymus, leukocyte, migratory cycle, chemotaxis, mitogens, NK

Introduction

The thymus of birds is colonized by different waves of prelymphoid cells during the embryogenesis as in mammals (Le Dourain *et al.*, 1977). In juvenile and adult birds it shows a structure similar to that of other vertebrates with well-defined cortex and medulla. In the avian thymus, the precursors of T lymphocytes mature by changing its morphology (Yasuda *et al.*, 1980) and expressing the characteristic molecular complexes of T lymphocytes in the cell surface (Fowlkes and Pardoll, 1989). Mature T lymphocytes recirculate into the peripheral blood and the lymphoid tissues yielding to the effectiveness of the immune response (Butcher, 1986).

In regard to the physiology of migratory birds, it is strongly affected by the migratory cycle, which induces significant changes mainly in muscle and fat tissues (Piersma *et al.*, 1999 and Schwilch *et al.*, 2002), but also in the pattern of endocrine function (Holberton and Able, 2000). Endocrine system has a well-known modulator role on the immune system (Saad, 1988), most evident in stressful situations (Sato and Glick, 1970) such as the long migratory flights. Moreover, migration involves exposure to variations in cold temperatures that modulate the immune response (Brenner *et al.*, 1999). Seasonal changes related to temperature and photoperiod have been reported even in the mammalian immune system (reviewed by Nelson and Demas, 1996). Another main factor in the immune response is the age, since juvenile birds have different patterns of response compared to adults (Traill *et al.*, 1983).

The present investigation addresses the study of the thymic cell function in female gulls *Larus ridibundus*. We have studied the most representative leukocyte functions such as mobility directed to a chemoattractant gradient (chemotaxis), lymphoproliferative response to mitogens and natural killer (NK)-like cell-mediated cytotoxicity. Since changes in the leukocyte functions have been reported in *L. ridibundus* throughout the migratory cycle (Muñoz and De la Fuente, 2003a and 2003b), the present study was performed after the migratory flight (post-migratory period) and when the gulls start to migrate again (premigratory period), searching for a putative effect on the immune response.

Materials and Methods

RPMI 1640 enriched with L-glutamine and HEPES, fetal bovine serum (FBS) and gentamicin were purchased from Gibco (Burlington, Ontario, Canada); Ficoll-Hypaque, N-formyl-Met-Leu-Phe (FMLP) and scintillation liquid from Sigma (St Louis, MO, USA); phytohaemagglutinin (PHA) and concanavalin A (Con A) from Flow Laboratories (McLean, VA, USA); transparent chemotaxis filters from Millipore (Bedford,

MA, USA); 96-well flat-bottomed and 96-well U-bottomed microtiter plates from Costar, (Cambridge, MA, USA); [^{3}H]-thymidyne (81 Ci mmol^{-1}) from Dupont (Boston, MA, USA); ^{51}Cr (1 mCi ml^{-1}) from New England Nuclear (Boston, MA, USA); harvest filter paper from Whatman Ltd. (Maidstone, UK); Phosphate-buffered saline solution (PBS) was prepared as follows: 123 mM NaCl, 11 mM Na_2HPO_4 and 3 mM KH_2PO_4. Phenol red-free Hank's solution was 5.5 mM glucose, 1 mM $MgCl_2$, 136 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.8 mM $MgHPO_4$, 0.5 mM KH_2PO_4, 0.4 mM Na_2HPO_4 and 4 mM $NaHCO_3$, adjusted to 7.4 pH.

Animals: The black-headed gull –*Larus ridibundus L., Laridae* – is a migratory species that arrives in Spain during autumn, and spends the winter there. At the end of this season the gulls migrate to northern Europe, returning to Spain in the following autumn. In the present study, female gulls were captured in the Spanish countryside, with legal permission from the Autonomous Government of Madrid (Spain). Sampling was performed just once per season. Thus, sampling in the post-migratory period was performed on the 1 November (when gulls had just arrived); and, in the premigratory period on the 3 March (when the gulls start to migrate). The size of the groups (juveniles and adults) was determined by the random capture technique. Juvenile gulls (1-2 years old) were identified by the different colors of the feathers in regard to adults (Bermejo *et al.*, 1986). Animals were anaesthetized with chloroform and decapitated according to the guidelines of the European Union Council Directives 86/6091 E.U.2.3.

Thymic Cell Isolation: Thymi were removed aseptically and stored at 4°C. They were taken to the laboratory and freed of fat, minced with scissors and gently pressed on a mesh screen to obtain a cell suspension in RPMI 1640 medium plus 10% FBS and 100 μg ml^{-1} gentamicin. The cell suspension was washed three times in PBS. The thymic mononuclear leukocytes obtained consisted of lymphocytes plus approximately 3% of monocytes, as was assessed by the cytocentrifugation of thymic cell suspensions. Cell viability was measured by using the trypan blue exclusion test showing a viability higher than 97%.

Chemotaxis Assay: The chemotaxis assay was carried out following a previously described technique (Muñoz and De la Fuente, 2003b). It consists in the use of chambers with two compartments separated by a nitrocellulose filter with a pore diameter of 3 μm; 300 μl aliquots of the leukocyte suspension (5 x 10^5 cells ml^{-1} in Hank's solution) were placed in the upper compartments of the chambers. Aliquots of the well-known chemoattractant agent for leukocytes FMLP at 10^{-8} M (Schubert and Müller, 1989) were placed in the lower compartments. For the controls, chemoattractant-free medium was used. The chambers were incubated for 3 h at 37°C. Filters were then fixed and stained, and cells migrating through the filter were counted in one-third of the lower face of the filter. Chemotactic indexes were calculated giving to the control a 100% value.

Lymphoproliferative Response to Mitogens: Thymic cells at a concentration of 2 x 10^5 cells 200^{-1} μl well^{-1} were seeded in 96-well plates in RPMI 1640 medium plus 10% FBS and 100 μg/ml gentamicin. 20 μl of mitogens or Hank's solution (controls) were also added to the wells. Mitogens used in this assay were 10 μg ml^{-1} PHA and 1 μg ml^{-1} Con A. Cells were maintained for 72 h in an incubator at 37°C and humidified atmosphere with 5% CO_2, and 0.5 μCi of [^{3}H]-thymidine well^{-1} were added 24 h prior to the collection of cells. Cells were harvested and [^{3}H]-thymidine uptake was measured in a Beckman LS 6000 scintillation counter (Palo Alto, CA, USA). Results were expressed as the percentage of [^{3}H]-thymidine uptake (cpm; counts per min), assuming a control uptake of 100%.

NK-like Cell-mediated Cytotoxicity Assays: The target cells used in the cytotoxicity assays were the human myeloid erythroleukemia cell line K-562. These assays were performed following the method previously described by Muñoz and De la Fuente (2003b). Briefly, target cells, maintained in RPMI 1640 medium, were labelled separately with ^{51}Cr (100 μCi/5 x 10^6 cells) for 90-120 min at 37°C, shaking every 15 min, and adjusted to 2.5 x 10^4 cells ml^{-1}. Thymic cells (effector cells) were adjusted to 2.5 x 10^6 cells m^{-1} l and added at 25:1 (effector : target cell ratio) for cytotoxicity assays.
U-bottomed microtiter plates with target and effector cells were incubated for 4 h at 37°C and then centrifuged for 10 min at 400 g. Aliquots of 100 μl were taken from the supernatants and counted in a gamma-counter (LKB, Uppsala, Sweden). The cytotoxicity percentage was calculated as follows: Cytotoxicity (%) = [(L - S) x 100] / (T - L), where L is the lysis measured by cpm released in the presence of effector cells; S is the mean of cpm spontaneously released by target cells incubated with medium alone; and T is the mean of total cpm released after incubating the target cells with 1:100 dilution of Triton X-100.

Statistical Analysis: Data are expressed as the mean ± SEM of the values from the number of experiments

performed in duplicate or triplicate as indicated in the corresponding figures. Data were evaluated statistically using the one-way ANOVA by addition of Kruskal-Wallis ranges for multiple comparisons, the minimum significance level being $P<0.05$.

Results and Discussion

The present work has been carried out to study a wide range of the main leukocyte functions that conform the cellular immune response, i.e. chemotaxis, proliferation and NK-like cell-mediated cytotoxicity, in gull thymic cells throughout the migratory seasonal cycle.

Thymic cells from females *L. ridibundus* were induced to migrate by FMLP (Table 1) as we found in previous studies with splenic leukocytes from both sexes and thymic cells from males of the same species (Muñoz and De la Fuente, 2003a and 2003b). It was also reported for leukocytes from chicken (Joshi and Glick, 1990). The lowest chemotaxis indexes were obtained in the post-migratory period for thymic cells from juveniles, while there were not differences in the chemotactic mobility of thymic cells from adults attending to the migratory period. These findings indicate a highest sensitivity to the stressful migratory flight in juveniles. It could be because there is a lowest maturation of the thymic cells in juveniles. In fact, there are studies performed in chicken reporting that leukocyte recirculation increases with the age of the animals (McCorkle *et al.*, 1990), may be due to a major production of mature leukocytes, or the action of different hormones affecting to the recirculation of leukocytes.

The mitogen-induced lymphoproliferative responses of gull thymic cells are shown as percentages in Fig. 1. Thymic cells from *L. ridibundus* showed a positive response to the mitogens assayed as we have previously reported in splenocytes and thymocytes from *L. ridibundus* (Muñoz and De la Fuente, 2003a and 2003b). This response has been also demonstrated in poultry as chickens (Holt, 1990 and Higgins, 1991). The mitogens PHA and Con A induced high proliferative percentages in thymic cells from *L. ridibundus* however there were not differences in the proliferative response in regard to the migratory period. No differences in the response associated to the age of the gulls were obtained even these differences have been reported in mammals (Kurakata *et al.*, 1989).

Thymic cells from *L. ridibundus* showed NK-like cell-mediated cytotoxicity (Fig. 2), as we have previously demonstrated in leukocytes from males of the same species (Muñoz and De la Fuente, 2003b). This activity has been also reported in chickens (Haddad and Mashaly, 1991). There were not differences in the cytotoxic response regarding the migratory period. Considering the age of the gulls, there was higher cytotoxic activity in thymic cells from juveniles than in those from adults during the premigratory period. This finding is similar to that reported in chicken, since the cytotoxic cell number is higher in juvenile animals as compared to adults (Lillehoj and Chung, 1992).

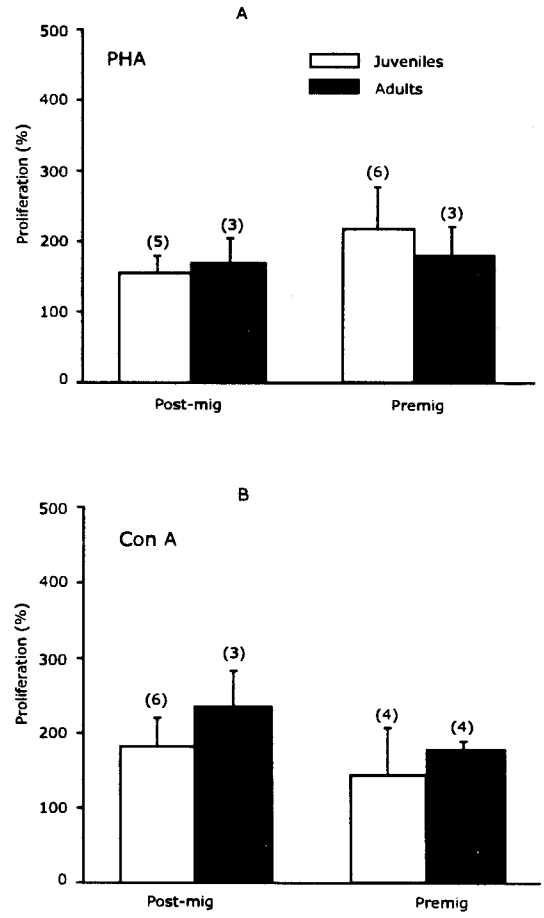

Fig. 1: Proliferative response of thymic cells to the mitogens PHA (A) and Con A (B) throughout the migratory cycle. The results are the mean ± SEM of the number of animals (in parentheses) on which the determinations were performed in triplicate

Summarizing, we have found that thymic cells from *L. ridibundus* share an immune response similar to that of poultry and mammals. The chemotactic mobility of thymic cells from juveniles is strikingly affected by the migratory seasonal cycle, suggesting a significant sensitivity to the stressful migratory flight in juveniles. Since the immuno-endocrine interactions modulate the response capacity of the organisms (Haddad and Mashaly, 1991) and induce dramatic effects on the

immune system, the action of hormones and different secretory factors could be also playing an important role in the modulation of the thymic cell response.

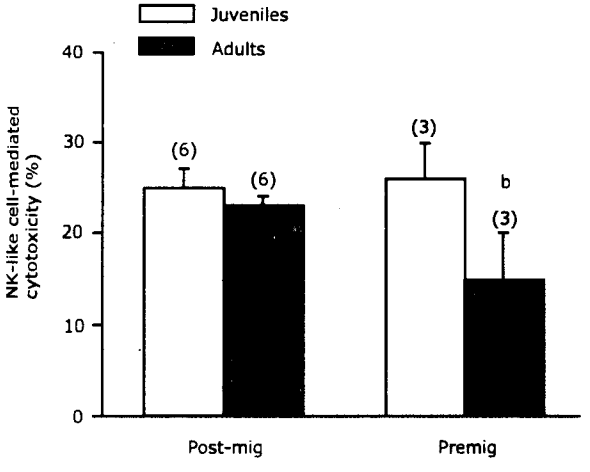

Fig. 2: NK-like cell-mediated cytotoxicity of thymic cells throughout the migratory cycle. The results are the mean ± SEM of the number of animals (in parentheses) on which the determinations were performed in triplicate. b $P<0.05$ versus juveniles

Table 1: Chemotaxis indexes of thymic cells in response to the chemoattractant N-formyl-Met-Leu-Phe (FMLP) throughout the seasonal migratory cycle of *Larus ridibundus*

	Post-migratory	Premigratory
Juveniles	$156_{(5)} \pm 10$	$200_{(3)} \pm 24^{a}$
Adults	$171_{(5)} \pm 19$	$186_{(3)} \pm 14$

Results are the mean ± SEM of the number of animals (in parentheses) in which the determinations were performed in duplicate. a $P<0.05$ versus post-migratory.

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