ABSTRACT Understanding the role of the pineal gland in regulating the immune response and the role of photoperiod in influencing pineal gland secretions are becoming increasingly important. The purposes of the present experiments were to investigate the effects of different photoperiod regimens on T- and B-lymphocyte activities in broiler chickens. Next, the influence of different photoperiod regimens on the responsiveness of lymphocytes to melatonin in vitro was examined. The effect of melatonin in vitro on lymphocyte activities was also studied, regardless of the photoperiod received. Finally, the effects of photoperiod on the profiles of different splenocyte cell types were investigated. To study the effect of photoperiod on lymphocyte activities, different photoperiod regimens were used. These were: constant lighting, 23 h light:1 h darkness; intermediate lighting, 12 h light:12 h darkness; and intermittent lighting, 1 h light:3 h darkness. Peripheral blood and splenic lymphocyte activities were tested at 3 and 6 wk of age by performing a mitogen cell-proliferation assay with a polyclonal T-cell mitogen, concanavalin A (Con A), and T-dependent B-cell mitogen, pokeweed mitogen (PWM). To study the effect of photoperiod on the responsiveness of lymphocytes to melatonin in vitro or the effect of melatonin in vitro on lymphocyte activities regardless of photoperiod received, lymphocytes from the chickens that were exposed to the different photoperiod regimens were incubated with mitogen and different concentrations of melatonin. To study the effect of photoperiod on profiles of different cell types, the percentages of splenocyte subpopulations from birds exposed to different photoperiods were determined using flow cytometry with CD4⁺, CD8⁺, CD3⁺, and B-cell markers. The results of these studies indicate that splenic T and B lymphocytes from 6-wk-old chickens grown in intermittent lighting had higher activities than those from chickens grown in constant lighting. Peripheral blood and splenic lymphocytes from chickens raised under constant lighting were more responsive to melatonin in vitro than those from chickens raised under intermittent lighting. This difference in response may be due to lower levels of melatonin in birds receiving constant lighting, making them more sensitive to melatonin in vitro. Melatonin in vitro enhanced the mitogenic response of peripheral blood T lymphocytes from 6-wk-old chickens, splenic T lymphocytes from 3-wk-old chickens, and splenic T and possibly B lymphocytes from 6-wk-old chickens. Finally, intermittent lighting increased the percentages of splenic CD4⁺, CD8⁺, and CD3⁺ cells but not B-cell subpopulations at 6 wk of age, presumably because of increased levels of melatonin in birds receiving intermittent lighting. Our results re-emphasize the importance of melatonin in regulating host immune response; this regulation could be accomplished through exposing broiler chicks to intermittent lighting.

(Key words: photoperiod, melatonin, lymphocyte proliferation, broilers)

2000 Poultry Science 79:18–25

INTRODUCTION

The neuroendocrine system plays a critical role in homeostatic regulation of the immune response (Blalock, 1989). The neuroendocrine and immune systems help an organism cope with changing environmental demands. Much experimental evidence indicates that the hypothalamo-pituitary-adrenal axis is activated in response to pathogens or environmental stressors. In recent years, melatonin, a neural hormone synthesized and secreted primarily by the pineal gland (Pelham et al., 1972; Pang and Ralph, 1975), has been suggested to have immunomodulatory roles. The involvement of melatonin in the establishment of a pineal-immune sys-

Received for publication October 5, 1998.
Accepted for publication September 1, 1999.
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Abbreviation Key: Con A = concanavalin A; FBS = fetal bovine serum; FITC = fluorescein isothiocyanate; IFN-γ = interferon-γ; LPS = lipopolysaccharide; PWM = pokeweed mitogen.
tem regulatory axis is currently of interest (Guerrero and Reiter, 1992; Liebmann et al., 1997).

Melatonin enhances the immune response and counteracts immunodeficiency states resulting from acute stress, viral diseases, aging, or drug treatment (Maestroni and Conti, 1993; Ben-Nathan et al., 1996). Melatonin also restores the impaired T-helper cell activity in immunodepressed mice and enhances antigen presentation to T cells through splenic macrophages (Pioli et al., 1993). Other roles of melatonin include the enhancement of natural killer cell activity (Del Gobbo et al., 1989), antibody-dependent cellular cytotoxicity (Giordano and Palermo, 1991), and the release of Interleukin 2 (IL-2), IL-6, and Interferon-γ (IFN-γ) (Garcia-Maurino et al., 1997). In mice, melatonin treatment in vivo increased concanavalin A (Con A)-induced lymphocyte proliferation but decreased lipopolysaccharide (LPS)-induced lymphoproliferation (Champney et al., 1997).

Recent approaches in understanding the mechanism of pineal gland regulation of immune function have focused on photoperiod or day length. Light-dark stimuli provide adequate environmental information necessary for physiological and behavioral adaptive changes. Pinealectomy seems to prevent photoperiodic responses in many mammalian species (Goldman, 1983). By manipulating photoperiod, melatonin production changes, reaching its maximum level at the midpoint of the dark phase and its minimum level at the midpoint of the light phase (Lynch, 1971). Therefore, the pineal gland appears to translate environmental cues into melatonin secretions that are necessary for daily and seasonal regulation of cardiopulmonary, reproductive, excretory, thermoregulatory, behavioral, and immune systems (Pang et al., 1996).

Most photoperiod studies on immune function to date show an increase in immune response in short compared with long days. Cockerels grown under constant lighting had a lower anti-SRBC titer than those grown under 12 h light:12 h darkness (Kirby and Froman, 1991). Total splenocyte numbers, macrophage counts, and splenic weights were significantly higher in hamsters exposed to a short photoperiod when compared with those of hamsters exposed to a long photoperiod (Brainard et al., 1985; Vaughan et al., 1987). In another study, a significant elevation in splenocyte proliferation to Con A was noticed in mice grown under short-day conditions compared with mice grown under long-day conditions (Demas et al., 1996).

The present study was conducted to address the effects of different photoperiod regimens on lymphocyte activities and on the responsiveness of lymphocytes to melatonin in vitro. The influence of melatonin in vitro on lymphocyte activities and the effects of different photoperiod regimens on the profiles of splenocyte subpopulations were also investigated.

**MATERIALS AND METHODS**

**Chickens and Experimental Design**

One-day-old commercial broiler chicks (Ross × Avian strain) were used in this study. The chicks were housed in floor pens and were randomly divided into different photoperiod treatments. Each treatment contained two replicate pens. The photoperiod treatments used were 23 h light:1 h darkness or constant lighting, 12 h light:12 h darkness or intermediate lighting, and 1 h light:3 h darkness or intermittent lighting. Food and water were provided ad libitum throughout the experiment. At 3 and 6 wk of age, peripheral blood was collected in heparinized syringes from the brachial vein. For extraction of spleens, birds were euthanized by cervical dislocation. Blood and spleen samples were obtained during the light period.

**Effect of Photoperiod on Mitogen-Induced Lymphoproliferation**

Blood and spleen samples collected from 10 chickens that were exposed to each of the above photoperiod regimens were assayed for lymphoproliferation in response to mitogen stimulation. The blood was mixed 1:1 with wash medium, RPMI 1640 with L-glutamine supplemented with penicillin (100 units/mL)/streptomycin (100 µg/mL), and spleens were pressed gently through 60-µm mesh screens using wash medium. The cell suspensions were then layered onto histopaque 1077 and were centrifuged at 220 × g for 30 min to separate the leukocytes. The white blood cell layer was removed and washed twice. Leukocytes were adjusted to 1 × 10^7 viable cells/mL in RPMI 1640 with 10% heat-inactivated fetal bovine serum (FBS). By using trypan blue exclusion, cell viability was determined to be ≥95% for suspensions of white blood cells and splenocytes.

Leukocytes were plated in triplicate cultures at 5 × 10^5 lymphocytes/well in 96-well, round-bottom plates. Each well contained leukocytes in 50 µL of medium. Fifty microliters of Con A was added at a final concentration of 12.5 µg/mL in the well, or 50 µL of PWM was added at a final concentration of 25 µg/mL in the well. The concentrations of Con A and PWM used were determined through preliminary experiments to give peak mitogenic response. Fifty microliters of RPMI 1640 with 10% FBS was also added to each culture. Cells were then incubated at 37 C in a humidified atmosphere of 5% CO₂ for 48 h. One microliter of ^3H-thymidine in 50 µL of medium was added to each culture. Cells were again incubated at 37 C under 5% CO₂ for 18 h to allow for ^3H-thymidine uptake. Cells were harvested onto glass-fiber filters using a cell harvester. The filters were placed in scintillation vials with scintillation fluid and were counted using a scintillation counter. Counts per minute were deter-
mained, and triplicate cultures were averaged; counts from cultures with no mitogens were subtracted.

**Effect of Photoperiod on the Response to Melatonin and the Effect of Melatonin on Mitogen-Induced Lymphoproliferation**

Leukocytes from the spleen and peripheral blood of 10 chickens exposed to each photoperiod regimen were plated separately with either Con A or PWM as described earlier. Fifty microliters of different concentrations of melatonin was also added to the cultures. The concentrations of melatonin added were 0 pg/mL, 50 pg/mL, 500 pg/mL, and 5 ng/mL for the peripheral blood samples and 0 pg/mL, 50 pg/mL, 500 pg/mL, 5 ng/mL, and 50 ng/mL for the spleen samples. Because splenocytes were consistently observed in our laboratory to be more active than peripheral blood leukocytes, an additional concentration of melatonin was added to the spleen samples to encompass the most effective in vitro concentrations. The concentrations of melatonin used were selected based on both physiological and supraphysiological concentrations in chickens. The melatonin solutions contained <0.01% ethanol necessary for dissolving the melatonin. Control cultures contained cells, mitogens, and 0.01% ethanol-supplemented medium. In addition, cultures with no melatonin and no mitogen were prepared to provide background counts. Triplicate cultures were averaged, and counts from the background cultures were subtracted from treatment counts (with mitogen and melatonin) or control counts (with mitogen and no melatonin). Percentage cell proliferation with melatonin added was calculated relative to the proliferation in the control cultures (with mitogen and no melanin). The counts for the control cultures were considered to be the baseline and were assigned a value of 100%. The following calculation was made:

\[
\text{Percentage cell proliferation} = \left( \frac{\text{treatment cpm} - \text{control cpm}}{\text{control cpm}} \right) \times 100 + 100.
\]

Because the two-way ANOVA revealed no photoperiod by melatonin interactions, only the main effects (photoperiod and melatonin in vitro) are reported in the results section. Therefore, when the effect of photoperiod on the response to melatonin in vitro is reported, percentage proliferation for all doses of melatonin is combined, and when the overall effect of melatonin in vitro is reported, percentage proliferation from all photoperiods is combined.

**Effect of Photoperiod on Profiles of Different Splenocytes**

Flow cytometry was performed on spleen samples at 3 and 6 wk from eight birds exposed to each photoperiod treatment. Cells were plated in 96-well, round-bottom plates using 100 µL of 1 × 10^7 cells/mL suspension. The plate was centrifuged at 220 × g for 20 min to pellet cells. The supernatant was decanted, and 100 µL of mouse anti-chicken monoclonal antibodies (obtained from H. Lillehoj, U.S. Department of Agriculture, Beltsville, MD 20705) against CD4, CD8, and CD3 were added. Information on these antibodies has been reported previously (Lillehoj et al., 1993). Furthermore, mouse anti-chicken monoclonal antibodies against Bu-1a and Bu-1b alloantigens (Veromaa et al., 1988) were used to label the B-lymphocyte populations. In addition, a pan-lymphocyte marker that labels all lymphocytes (K55) was also used as previously described by Chung et al. (1991). Finally, a mouse anti-human T-lymphocyte monoclonal antibody, T3-3A1(HB2) from American Type Culture Collection (ATCC), was used as a negative control to determine the nonspecific binding for the primary antibody as described previously by Trout and Lillehoj (1996). Monoclonal antibodies were added to different cultures and were incubated at 4°C for 30 min. The cultures were washed twice with a staining medium consisting of PBS and 0.1% sodium azide (NaNO₃). One hundred microliters of 1:100 anti-mouse IgG, antigen binding fragment (Fab)-specific fluorescein isothiocyanate (FITC) conjugate was added to each culture and was incubated at 4°C for 30 min. Cells were washed twice with staining medium and then fixed with 200 µL of 1% paraformaldehyde solution in PBS. Fluorescence was analyzed using a Coulter XL. Percentage fluorescent-positive live cells were adjusted using a pan-lymphocyte marker (K55) and a negative control marker (HB2). As the nonspecific binding of the secondary FITC-labeled antibody was consistently <0.2%, it was not included in the calculation. Therefore, the following calculation was made:

\[
\text{Percentage fluorescent-positive live lymphocytes} = \left( \frac{\text{percentage fluorescent-positive live cells} - \text{percentage HB2-positive live cells}}{\text{percentage K55/100}} \right)
\]

**Statistical Analysis**

The general linear models procedure of SAS® software was used to analyze data with either a one-way analysis of variance (when the effect of photoperiod on lymphoproliferation was the main effect) or two-way analysis of variance (when the effect of photoperiod on the response of lymphocytes to melatonin, and the effect of melatonin in vitro on lymphoproliferation were the main effects) (SAS Institute, 1996). Means were separated using Duncan’s multiple-range test with significance set at \( P < 0.05 \).

**RESULTS**

**Effect of Photoperiod on Mitogen-Induced Lymphoproliferation**

No significant differences due to different photoperiod regimens were found in lymphocyte proliferations from...
Peripheral blood of 3- or 6-wk-old chickens (Table 1) or from splenic (Table 2) lymphocytes of 3-wk-old chickens. This result occurred in response to either Con A or PWM. However, at 6 wk of age, there were significant differences in chicken splenocyte proliferation among the three photoperiods (Table 2). In response to Con A, T lymphocytes from birds grown in intermittent lighting had a significantly higher proliferative response than birds grown in both intermediate and constant lighting. In response to PWM, lymphocytes from birds grown in intermittent and intermediate lighting had significantly higher proliferative responses than birds grown in constant lighting.

**Effect of Photoperiod on the Response to Melatonin In Vitro**

There was no significant effect of photoperiod treatment on the proliferative response of peripheral blood lymphocytes from 3-wk-old chickens to either mitogen when the cells were incubated with melatonin in vitro (data not shown). When lymphocytes from 6-wk-old chickens were used, Con A-stimulated peripheral blood T lymphocytes from birds grown in constant lighting were significantly more responsive to melatonin in vitro when compared with birds grown in either intermittent or intermediate lighting (Figure 1). Furthermore, Con A-stimulated, splenic T lymphocytes from 3-wk-old chickens raised in constant light showed significantly higher proliferation in response to melatonin in vitro than splenic T lymphocytes from chickens raised in intermittent light (Figure 1). In addition, Con A-stimulated, splenic T lymphocytes from 6-wk-old birds raised in constant lighting showed a significantly higher percentage proliferation in response to melatonin in vitro than splenic T lymphocytes from birds raised in either intermittent or intermediate lighting (Figure 1). However, PWM-stimulated peripheral blood lymphocytes from 6-wk-old chickens and splenocytes (from 3- and 6-wk-old chickens) grown under constant lighting showed no significant difference in proliferative response to melatonin in vitro compared with lymphocytes from chickens grown under intermittent lighting (Figure 1). Pokeweed mitogen-stimulated peripheral blood lymphocytes from 6-wk-old chickens and splenocytes from 3-wk-old chickens that were raised in intermediate lighting showed a significant decrease in proliferative response to melatonin in vitro compared with lymphocytes from chickens raised in intermittent light. Furthermore, in the 6-wk-old chickens, intermediate lighting significantly decreased the proliferative response of peripheral blood and splenic PWM-stimulated lymphocytes to melatonin in vitro compared with constant light.

**Effect of Melatonin In Vitro on Mitogen-Induced Lymphoproliferation**

At 3 wk of age, melatonin in vitro had no significant effect on mitogen-induced peripheral blood T- or B-lymphocyte proliferation (Figure 2). However, when peripheral blood lymphocytes from 6-wk-old chickens or splen-
FIGURE 1. Effect of photoperiod on lymphocyte response to melatonin in vitro (doses of melatonin combined) using mitogen-stimulated peripheral blood lymphocytes from 6-wk-old broiler chickens (A); mitogen-stimulated splenocytes from 3-wk-old broiler chickens (B); and mitogen-stimulated splenocytes from 6-wk-old broiler chickens (C). Values are means ± SEM. Values with no common letters are significantly different (P < 0.05). Concanavalin A (Con A) and pokeweed mitogen (PWM) are represented by lower and upper case letters, respectively. (A) n = 6 – 10; (B) n = 5 – 9; (C) n = 8. L = hours of light; D = hours of darkness. *Percent Cell Proliferation = (Treatment cpm – control cpm)/control cpm) × 100 + 100.

FIGURE 2. Effect of melatonin in vitro regardless of photoperiod (different photoperiod treatments were combined) using mitogen-stimulated peripheral blood lymphocytes from 3-wk-old broiler chickens (A); mitogen-stimulated peripheral blood lymphocytes from 6-wk-old broiler chickens (B); mitogen-stimulated splenocytes from 3-wk-old broiler chickens (C); and mitogen-stimulated splenocytes from 6-wk-old broiler chickens (D). Values are means ± SEM. Values with no common letters are significantly different (P < 0.05). Concanavalin A (Con A) and pokeweed mitogen (PWM) are represented by lower and upper case letters, respectively. (A) n = 6 – 8; (B) n = 6 – 10; (C) n = 5 – 9; and (D) n = 8. *Percent Cell Proliferation = (Treatment cpm – control cpm)/control cpm) × 100 + 100.
ocytes from 3-wk old chickens were used, melatonin in vitro significantly increased peripheral blood T but not B-lymphocyte, mitogen-induced proliferation (Figure 2). Finally, by using the splenocytes from 6-wk-old chickens melatonin in vitro was found to enhance mitogen-induced T- and B-lymphocyte proliferation (Figure 2).

All enhanced mitogen-induced lymphocyte proliferations reported above were due to melatonin in vitro when compared with lymphocyte proliferations that were due to mitogen stimulation but with no melatonin added.

**Effect of Photoperiod on Profiles of Different Splenocytes**

There was a significant effect of photoperiod on the percentages of different cell populations for both ages. At 3 wk of age, splenocytes cultured from birds in intermediate light had a significantly lower percentage of CD4+ cells but a significantly higher percentage of B cells compared with splenocytes from birds in constant light (Figure 3). At 6 wk of age, spleens cultured from birds in intermittent lighting had significantly higher percentages of CD4+, CD8+, and CD3+ cells than birds in constant lighting. However, no significant difference was noticed in the B-cell population between photoperiod treatments (Figure 4).

**DISCUSSION**

In the present study, intermittent lighting was found to enhance mitogen-induced splenocyte proliferation in 6-wk-old broiler chickens when compared with either constant or intermediate lighting regimens. This finding is supported by Dobrowsolska and Gromadzka-Ostrowska (1984), who reported that splenocytes extracted from male deer mice that received 8 h light:16 h darkness/day for an 8-wk period exhibited greater proliferation to Con A than splenocytes from mice that received 16 h light:8 h darkness/day. Our results and the results by Dobrowsolska and Gromadzka-Ostrowska (1984) indicate that increasing periods of darkness stimulate mitogen-induced splenocyte proliferation. Futhermore, Demas and Nelson (1996) showed that deer mice grown in short days exhibited larger spleen size and higher antibody titers than deer mice maintained in long days.

There are two possible explanations for the effects of photoperiod on mitogen-induced lymphocyte proliferation. Champney et al. (1997) suggested that melatonin can disproportionately alter the number of blood and splenic T and B lymphocytes, or it can modify the intrinsic mitogenic activity of each lymphocyte. The results from the present study indicate that both a modification of mitogenic activity and change in cell populations might have occurred. First, the intermittent photoperiod enhanced the percentages of CD4+, CD8+, and CD3+ splenocyte subpopulations. Second, intermittent lighting greatly enhanced splenocyte proliferation. Whether the enhanced splenocyte proliferation can be attributed to an increase in cellular activity or simply to an increase in percentages of certain cell type(s) is yet to be determined. However, results from our experiment imply that melatonin in vivo can change splenocyte profiles and also may alter cellular activity.

In addition to photoperiod having a direct effect on mitogen-induced splenocyte proliferation and the pro-
portions among splenocyte populations, it also enhanced the sensitivity of lymphocytes to melatonin stimulation in vitro. Lymphocytes obtained from birds grown in 23 h light:1 h darkness were more responsive to melatonin stimulation than lymphocytes from birds grown in 1 h light:3 h darkness. Colombo et al. (1992) have suggested that spleen cells obtained at night have a high sensitivity to melatonin, whereas cells obtained in the morning have a low sensitivity because of up- and down-regulation of receptors, respectively. A low level of melatonin in chickens grown under constant lighting as reported by Pelham et al., (1972) could allow for an up-regulation of melatonin receptor expression. Lymphocytes from birds in constant lighting, therefore, are more sensitive when incubated with melatonin. However, lymphocytes from birds raised in intermittent lighting are less sensitive to melatonin.

In the present study, melatonin in vitro enhanced peripheral blood and splenic lymphocyte proliferation after stimulation with Con A or PWM. These results are similar to those found by Champney et al. (1997), who reported that hamsters injected with melatonin exhibited an increase in splenocyte proliferation when stimulated with Con A. The mechanism of lymphocyte proliferation caused by melatonin can possibly occur through enhanced synthesis and secretion of IL-2, IL-6, and IFN-γ as reported by Garcia-Maurino et al. (1997) using human circulating CD4+ cells. However, Konakchieva et al. (1995) suggested that melatonin in vitro decreases human lymphocyte proliferation. Furthermore, Rogers et al. (1997) suggested that melatonin alone did not significantly affect lymphocyte proliferation. Differences in the concentration of mitogen may have an effect on melatonin enhancement of mitogenic proliferation.

Alterations in mitogenic activity by melatonin in vitro showed peripheral blood and splenic T lymphocytes to be more responsive than B lymphocytes. Melatonin receptors found on human T lymphocytes have a higher binding affinity than B lymphocytes (Gonzalez-Haba et al., 1997). This suggests that T lymphocytes are the preferred target cell of melatonin action. However, stimulation of splenic B lymphocytes at 6 wk of age, in the present study, caused an increased mitogenic activity to PWM. It is known that PWM is a T-cell-dependent B-cell stimulator that has been shown to stimulate proliferation of chicken B cells, as well as a small population of chicken T cells (Vainio and Ratcliffe, 1984). Weber (1973) reported that PWM induced a significant T-cell response, in addition to a B-cell response, in the spleen. Therefore, splenic B-lymphocyte proliferation at 6 wk, in the present study, may be due to the stimulation of the T-cell subsets by PWM.

In summary, our results indicate that the intermittent photoperiod regimen, indirectly through melatonin, enhances splenic immune functions of broiler chickens when compared with constant lighting. These results suggest the important role that photoperiod plays in affecting the immune response. Furthermore, lymphocytes from birds receiving constant lighting are more sensitive to melatonin in vitro than lymphocytes from birds receiving intermittent lighting. Melatonin in vitro also stimulates mitogen-induced lymphocyte proliferation. Exogenous melatonin may, therefore, be very effective in stimulating the immune response. It is concluded that melatonin can modify immune function both in vivo and in vitro. Further examination of the mechanism through which melatonin acts in vitro can help to understand the role of melatonin in vivo on neuroimmunomodulation.

ACKNOWLEDGMENTS

The authors wish to thank D. Weinstock (Department of Veterinary Science, Penn State University) for providing Bu-1a and Bu-1b monoclonal antibodies. The authors would also like to thank G. Hendricks, III (Department of Poultry Science, Penn State University) for his assistance in reviewing our manuscript.

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