ヒナのマクロファージのIgG-Fcレセプター陽性率,スーパーオキサイド産生および抗原提示能におけるリズム性の存在

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Presence of Diurnal or Circadian Rhythms in IgG-FcR Positive Rate, Superoxide Production and Antigen Presenting Activity of Macrophages in Chicks

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The rhythmic variations during a day in superoxide (O_2^-) production, Fc receptor (FcR) positive rate and antigen-presenting activity of abdominal macrophages were investigated in 6- to 9-week-old White Leghorn chicks. It was evident that these features of macrophages showed clear rhythmic patterns in a day. The rhythmic pattern of O_2^- production showed a peak during light period in both zymosan-stimulated and unstimulated macrophages. The pattern was not synchronized with those observed in bactericidal activity of chicken abdominal macrophages. FcR positive rate of abdominal macrophages showed a peak under light and dark conditions, respectively. This pattern synchronized almost completely with the rhythm of bactericidal activity, but rhythmic correlations were not observed between FcR positive rate and phagocytic activity. Macrophages added to the lymphocyte culture accelerated lymphocyte activity by presenting antigens to them, as shown by SI values exceeded 1.0. Antigen-presenting activity of macrophages exhibited a rhythm with two peaks during the light period. The rhythmic pattern was not synchronized with the activation pattern of *in vitro* phytohemagglutinin (PHA) -stimulated chicken peripharal lymphocytes. The formation of the rhythm in T cell activation possibly depends on other factors or intrinsic change in T cell activity.

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Key words: diurnal or circadian rhythm, chicken macrophage, Fc receptor, superoxide, antigen-presenting activity

Introduction

It has been well known that many features of immunological function in humans (ESKOLA *et al.*, 1976) and rodents (FERNANDES *et al.*, 1976 ; POWNALL and KABLER, 1978 ; KNYSZYNSKI and FISHER, 1981) show quantitative and qualitative fluctuations during a day. In avian species, the investigation and elucidation of rhythmicity in immune functions are important for the establishment of optical timing of vaccination programs for several infective agents and administration programs for drugs acting upon the immune systems. In the chicken, leukocyte counts (GLICK, 1960), and activities of B cells and T cells in several specific immune functions (STINSON*et al.*, 1980 ; CAHYANINGSHI *et al.*, 1990 a,b), and of heterophils (CAHYANINGSHI *et al.*, 1990 c) with respect to their phagocytic and bactericidal activities have been shown to have clear rhythmic variations.

With regard to the rhythm of chicken mononuclear phagocytes, peripheral blood monocyte count, and phagocytic and bactericidal activities of abdominal macrophages

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show clear rhythmic fluctuations (KONDO *et al.*, 1992). Macrophages are concerned not only in the nonspecific immune functions in company with polynuclear phagocytes by exerting their phagocytic and bactericidal mechanisms but in the specific immune functions by presenting modified antigenic substances for helper T cells (SCHWARTS *et al.*, 1978 ; UNANUE, 1980). In the present study, rhythmic fluctuations in the IgG-Fc receptor positive rate and superoxide production of chicken abdominal macrophages were investigated in order to determine wheter they are involved in the formation of phagocytic and bactericidal activities of chicken macrophages. In addition, the rhythm of antigen-presenting activity in chicken macrophages was investigated in order to determine whether it is related to the rhythmic fluctuation of lymphocyte activities.

Materials and Methods

Chicks and Laboratory Conditions

Male chicks of the White Leghorn Julia Strain, 6 to 9 week old were used. Ten days before each experiment, the chicks were transferred to a closed room (1.8×2.2×2 m), and then maintatined on, and adapted to, an artificial light condition (70 to 100 lux) of 14 hours light (lights on from 06.00~20.00 hour) and 10 hours darkness and room temperature at 23±1℃. The chicks were provided *ad libitum* access to a commercial ration (crude protein above 14%, metabolizable energy above 2,850 kcal/kg) and water throughout the experiment.

Superoxide Production Assay

At intervals of 4 hours for 24 hours (07.00, 11.00, 15.00, 19.00, 23.00, and 03.00 hour), macrophage-rich peritoneal exudates were collected from the abdominal cavities of chicks pre-injected with Sephadex G-50 Fine (Pharmacia) saline suspension as a stimulator of macrophage induction, and then macrophages in each exudate were purely isolated using a macrophage adherent procedure as described elsewhere (KONDO *et al.*, 1992). Zymosan A (Sigma) (0.01g) was opsonized by incubating with 5 m*l* of 10% fresh chicken serum (Mg, Ca (−) Hank's blalnced salt solution) (HBSS), at 37℃ for 20 min. After centrifugation (1800×G, 10 min, 4℃), zymosan A was resuspended into modified Krebs-Ringer phosophate buffer solution (KRP). Superoxide (O_2^-) production assays were performed on unstimulated (resting) macrophages and zymosan-stimulated (phagocytosing) macropahges. For resting samples, 0.4 m*l* of macrophage suspension (5×10^5/m*l* of HEPES-RPMI 1640 culture medium), 0.4 m*l* of 80 μM cytochrome C (Sigma) solution, and 0.2 m*l* of KRP, or, for phagocytosing samples, equal volumes of macrophage suspension and cytochrome C solution, 0.1 m*l* of opsonized zymozan A, and 0.1 m*l* of KRP were mixed. The mixtures were incubated under constant rotation at 37℃ for 1 hour. After centrifugation (10 min, 1800×G, 0℃), supernatants were determined with a spectrophotometer (UV-140-02, Shimazu) at 550 nm. Optical densities at each determination points were regarded as O_2^- values produced by macrophages. Stimulation index (SI) was calculated by dividing the zymosan-stimulated value by unstimulated value at each determination point.

IgG-FcR positive Rate Assay

Fc receptor (FcR) positive cells have been detected with heat-aggregated Ig in chickens (NOWAK *et al.*, 1982).

Chicken serum Ig was precipitated with sodium sulfate. The precipitate was dialyzed, and fractionated with a Sephadex G-200 (Pharmacia) column in 0.16 M borate buffer, pH 8.2. The second elution peak containing mostly IgG was collected, and concentrated with 20% polythylene glycol. The IgG precipitin was labeled with fluorescein isochiocyanate (FITC, Sigma) as described elsewhere (KONDO *et al.*, 1987). The FITC labeled-IgG was heat aggregated at 63℃ for 20 min and then centrifuged at 400×G for 10 min to remove large aggregates. Final concentration of IgG solution was 1.0 to 1.5 mg/m*l*. The solution was stored at −20℃ until use.

Abdominal macrophages collected as previously described at each determination point (1.5×10^6/0.2 m*l* of HBSS) were mixed with 0.2 m*l* of FITC labeled-chicken IgG, and incubated for 90 min at 4℃. After washing three times, the cell were resuspended with HBSS. The number of stained cells in each cell suspension was counted under a fluorescence microscope (FM-200A, Tiyoda), and the positive rate was calculated from the number of stained cells and total cell number counted under visible light condition (>200 cells).

Antigen Presenting Assay

An antigen presenting assay on chicken macrophage was performed by modified method of FARR *et al.* (1979). Briefly, *listeria monocytogenes* (child origin) supplied through the courtesy of Ueno Fine Chemical Industry, Ltd (Osaka, Japan) was cultured in YCC broth (Eiken) for 24 hours at 37℃. After cultivation, the number of living organism was counted, and adjusted to 1×10^5/m*l* of HBSS. Living organisms were killed by suspending in 5% phenol for 30 min, and the number of sterilized organism was counted and adjusted to 1×10^5/m*l* of HBSS.

At each determination time, 0.05 m*l* of living *listeria monocytogenens* suspension (5×10^4) was injected into wing vein to sensitize chicks against *listeria* antigen. At the same times of 4 days after the senstization, chicks were injected with Sephadex G-50 Fine intraabdominally. Five days after *listeria* injection, 2 m*l* of heparinized blood was sampled from the wing vein of chicks at each determination time. After centrifugation, buffy coat (leucocyte layer) was collected and suspended in HBSS. The leukocyte suspension was placed on percoll (Pharmacia) solution (specific gravity : 1.065) and centrifuged at 400×G for 15 min at 4℃. Lymphocytes at the surface of percoll solution were collected and suspended into 0.32% NaCl solution to lyse contaminated erythrocyte. After washing twice, the cells were suspended into HEPES-RPMI 1640 culture medium. After incubation in a CO_2 incubator (5% CO_2, 95% air) for 30 min, the number of cells was adjusted to 1×10^7/m*l*. Immediately after the blood sampling (within 5 min), abdominal macrophages were collected from same chicks (24 hours after Sephadex G-50 Fine injection), and adjusted to 2×10^4/m*l* and 2×10^5/m*l* with HEPES-RPMI 1640 culture medium. Five tenths milliliter of lymphocytes suspension (1×10^6 or 1×10^5 cells) and 0.5 m*l* of macrophage suspension (1×10^4 or 1×10^5 cells) derived from same individual, and 0.5 m*l* of *listeria monocytogenes* suspension (5×10^7 bacterias) were

mixed. For control, mixtures without macrophages were prepared. The mixtures were incubated in a CO_2 incubator (5% CO_2, 95% air) for 48 hours at 36℃. Each culture was added with 0.1 m*l* of methyl-^{3}H-thymidine (37 kBq, Amersham) and incubated for an additional 24 hours. After incubation, the mixtures were centrifuged at 1800×G for 10 minutes and washed with 3% acetic acid solution three times by repeated centrifugation. After the final centrifugation, cell sediment was added with 0.3 m*l* of sample solubilizer (Amersham), 0.015 m*l* of acetic acid, and 10 m*l* of toluene-scintilation solution. DPM of each sample was counted by scintilation counter (Aloka). Stimulation index was calculated as antigen-presenting activity on dividing the value obtained from macrophages-containing culture by that from culture not containing macrophages at same determination time.

Statistical methods

Duncan's new multiple range test was used to determine significances between from 07.00 hour to the next day at 07.00 hour. All significant differences were determined 1% and 5% levels. All results are given as means and standard errors.

Results

1. O_2^- production by abdominal macrophage

O_2^- production of abdominal macrophages was stimulated by addition of zymosan, suggesting that the bactericidal activity of macrophage was activated by zymosan (Fig. 1). The rhythms of O_2^- production by zymosan-stimulated and unstimulated macrophages showed a peak at 19.00 hour, respectively (Fig. 1). The values at peaks were significantly different from those at 11.00 hour ($P<0.01$) and 23.00 hour ($P<0.01$) in unstimulate macrophage, and that at 11.00 hour ($P<0.05$) in stimulated macrophage.

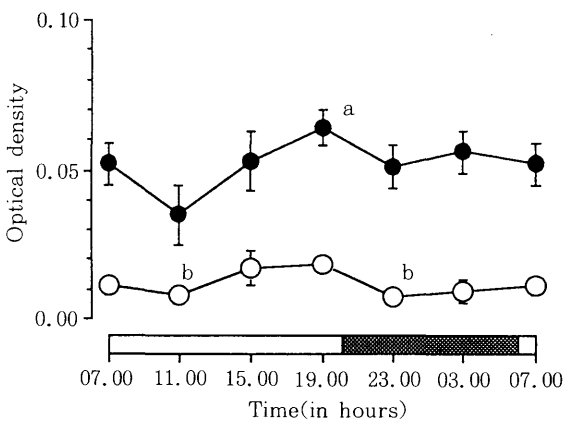

Fig. 1. Rhythmic patterns of O_2^- production by zymosan stimulated (—●—) and unstimulated (—○—) abdominal macrophages of chicks
Vertical bars represent standard error. Six chicks were used at each determination. a=Significantly different from the value at 11.00 hour ($P<0.05$). b=Significantly different from the value at 19.00 hour ($P<0.01$).

Stimulation index (stimulated/unstimulate) under dark condition (9.83±3.62 at 23.00 hour and 13.99±6.48 at 03.00 hour) tended to be higher than that under light condition (3.92±0.71 at 19.00 hour to 5.41±1.26 at 07.00 hour) (Fig.2), suggesting that reactivity of oxydative bactericidal mechanism in chicken macrophage against foreign substances is higher under dark condition.

2. IgG-FcR positive rates of abdominal macrophage

The rhythmic pattern of IgG-FcR positive rate showed a peak under light [at 11.00 hour (98.2±0.7%) to 15.00 hour (99.7±0.2%)] and dark condition [at 23.00 hour (96.0±2.5) to 03.00 hour (96.6±0.8)], respectively (Fig.3). During those periods, a great majority of macrophages exuding into abdominal cavity were IgG-FcR positive cells. The values

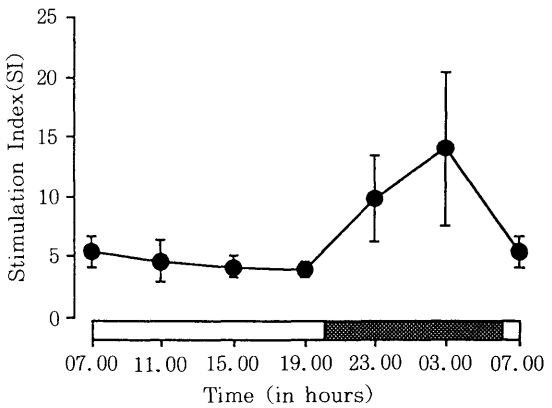

Fig. 2. Rhythmic pattern of stimulation index (SI) for O_2^- production by abdominal macrophages of chicks
Vertical bars represent standard error. Six chicks were used at each determination.

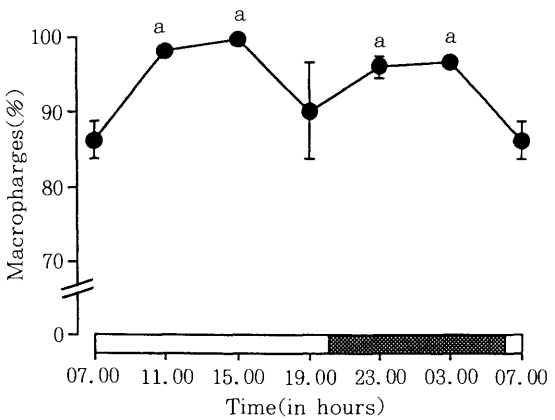

Fig. 3. Rhythmic pattern of IgG-FcR positive rate in abdominal macrophages of chicks
Vertical bars represent standard error. Six chicks were used at each determination. a=Significantly different from the value at 07.00 hour ($P<0.01$).

at the two peaks were significantly different ($P<0.01$) from that at 07.00 hour (86.2 ± 2.5).

3. Antigen-presenting activity of abdominal macrophage

Lymphocyte activities against *listeria monocytogenes* estimated by ^{3}H-thymidine uptake (DPM) were higher during darkness (23.00 hour and 03.00 hour) than during light conditions (07.00 hour to 19.00 hour) in all three cultures. They showed a peak at 23.00 hour, respectively (Fig.4). The DPM in macrophage-containing cultures were higher than that in control culture at all determination times, and the value obtain from the culture containing large numbers of macrophage (1×10^5 cells) tended to be higher than that containing small number of macrophage (1×10^4 cells) (Fig.4).

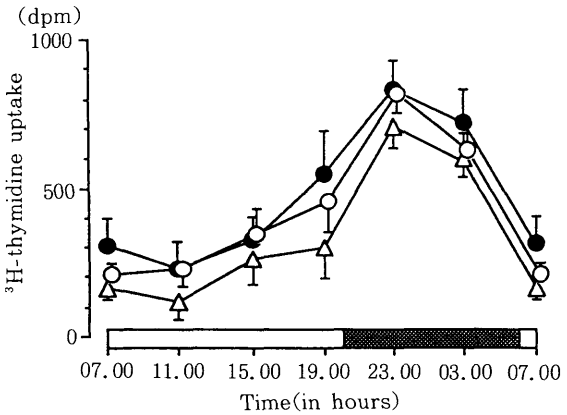

Fig. 4. Rhythmic patterns of thymidine uptake by peripheral lymphocytes with (—●— : 10^5 cells, —○— : 10^4 cells) or without abdominal macrophages (—△—) in chicks
Vertical bars represent standare error. Six to seven chicks were used at each determination.

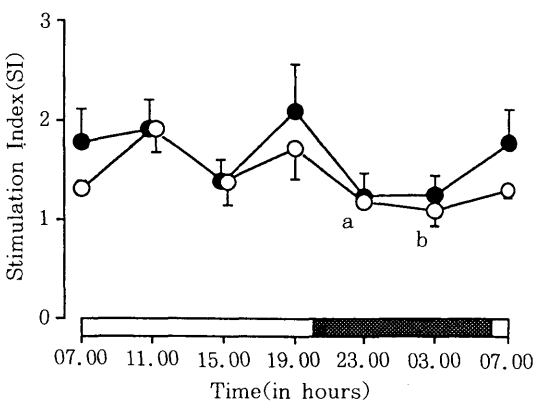

Fig. 5. Rhythmic patterns of antigen-presenting activity of chicken abdominal macrophages (—●— : 10_5 cells, —○— : 10^4 cells)
Vertical bars represent standard error. Six to seven chicks were used at each determination. a=Significantly different from the value at 11.00 hour ($P<0.05$). b=Significantly different from the value at 11.00 hour ($P<0.01$).

Stimulation index exceeded 1.0 in both the macrophage-containing cultures at all determination times, suggesting that macrophages in the cultures amplified lymphocyte activities by presenting *listeria* antigen to them. Moreover, SI values in the culture containing 1×10^5 macrophages were higher than that containing less number of macrophages at all determination times other than 07 : 00 hour (Fig.5). SI values obtained from both cultures showed a deppression during dark period (23 : 00 hour to 03 : 00 hour), respectively. In the macrophage-less culture, the values at 2300 hour (1.18 ± 0.07) ($P<0.05$) and 03 : 00 hour (1.09 ± 0.14) ($P<0.01$) were significantly lower than that at 11.00 hour (2.07 ± 0.24). Thus, antigen presenting activity of macrophages appeared to be higher during light period than dark period.

Discussion

The present experiment demonstrated that presence of rhythms on superoxide production, FcR positive rate, and antigen-presenting activity of chicken abdominal macrophages. It was also demonstrated that activities of isolated chicken macrophages show some variation *in vitro* during a day, as supposed in previous experiment (KONDO *et al.*, 1992).

Superoxide production in macrophages showed a peak during light period in both condition of zymosan-stimulating and unstimulating. O_2^- produced by polynuclear and mononuclear phagocytes has been closely related to their bactericidal activities (BABIOR *et al.*, 1973). It is thus reasonable that O_2^- productions were higher in phagocytosing macrophages. However, the variation pattern of O_2^- production in macrophages did not synchronize with the patterns observed in bactericidal activity of chicken macrophages, which showed two peaks (KONDO *et al.*, 1992). This is the same situation as isolated chicken heterophils (CAHYANINGSIH *et al.*, 1990c). Likewise, no relation was apparent between reactivity in O_2^- production estimated by SI value, and bactericidal activity in chicken macrophages. Phagocytes have been known to produce several oxygen compounds other than superoxide, such as hydrogen peroxide, hydroxyl radical, and singlet oxygen for their intracellular killing (W, 1987). Oxygen-independent systems have been also related to intracellular killing of phagocytes (ELSBACH and WEISS, 1981). Therefore, the rhythmic patterns of bactericidal activity and O_2^- production are not necessarily the same because the bactericidal activity of macrophages seems to be total activities in these agent correlated each other.

It is considered that rhythmic variation (in a day) observed in FcR positive rate of chicken peritoneal macrophages means that the replacement of macrophage populations are considerably frequent in the peripheral as shown in suface Ig-positive (sIg^+) B cell of human peripheral blood (ABO *et al.*, 1978) and sIg^+ and B cell antigen-positive (B+) B cell of chicken peripheral blood (CAHYANINGSIH *et al.*, 1990). It has been known that FcR on the surface of phagocytes acclerates not only the phagocytic activity by a process known as zippering in company with C3R (WERB, 1987) but the bactericidal activity by affecting it (ROSENTHAL, 1980). The FcR positive rate of chicken abdominal macrophages synchronized almost completely with their bactericidal activity, but rhythmic correlations were not observed between FcR positive rate and phagocytic

activity in chicken macrophages. Therefore, it is assumed that increase in FcR^- population in chicken macrophages is strongly related to bactericidal activity of chicks.

It has been well known that macrophages (as a kind of accessory cell) are able to activate helper T cells by processing and presenting it to them (SCHWARTS, 1978 ; UNANUE, 1980). The SI values (DPM of macrophage-containing culture/DPM of control culture) exceeded 1.0 in the present experiment confirm to this ability of macrophages also in chicken. Phytohemagglutinin (PHA)-stimulated peripheral blood T cell activity *in vitro* in chicks has been reported to show clear monophasic variation pattern, which is higher during the dark period (CAHYANINGSIH *et al.*, 1990b). However, antigen-presenting activity of macrophages estimated by SI value showed a rhythm that has two peaks during the light period and is low during darkness. Therefore, involvement of macrophages in the rhythm formation of T cell may be contradictory in chickens. The PHA-stimulated T cell acivity *in vitro* showed a rhythm elevated during dark period regardless of the presence of macrophages in the culture. Accordingly, formation of rhythm in T cell activation is considered to be attributed principally to the variation of T cell activity in a day (intrinsic fluctuation of T cell activity), and it is difficult to assume that accesory functions of macrophages are involved in rhythm formation of T cell activation.

It is assumed that there are close relations between macrophage activities and melatonin, which showes circadian release and which is related to leucocyte proliferation rhythm in humans *in vivo* (KUCI *et al.*, 1988). In this case, fluctuated melatonin levels in blood synchronized with light condition may influence to immunological events. However, it may be difficult to conclude that a cause of *in vitro* rhythmic variations in chicken macrophage activities observed in the present or early experiments (KONDO *et al.*, 1992) is attributed to the fluctuation of malatonin level *in vivo*. *In vitro* fluctuations might originated from intrinsic rhythmic variation of macrophage activities. Thus, it remains unclear whether *in vitro* fluctuations in macrophage activities are diurnal or circadian.

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ヒナのマクロファージのIgG-Fcレセプター陽性率，スーパーオキサイド産生および抗原提示能におけるリズム性の存在

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ヒナの腹腔マクロファージにおけるスーパーオキサイド（O_2^-）産生能，IgG-Fcレセプター（FcR）およびT細胞への抗原提示能について，一日の時間的経過を調査して，それらのリズム性を追究した。これらのマクロファジーの活性にはそれぞれ明確な日内変動が認められた。O_2^-産生はザイモザン刺激および無刺激マクロファージのいずれにおいても明期にピークを示す変動を示した。この変動パターンはニワトリマクロファージの静菌活性の変動パターンとは一致しなかった。FcRの陽性率は明期と暗期にそれぞれのピークを持つ変動を示した。このパターンはマクロファージの静菌活性の変動とほぼ完全に一致したが，貪食活性の変動とは一致しなかった。リンパ球とリステリア菌の混合培養にマクロファージを添加すると，リンパ球によるチミジンの取り込みが上昇し，マクロファージによるTリンパ球への抗原提示が観察された。マクロファージの抗原提示能は明期に二つのピークを持つ変動を示した。この変動パターンはフィトヘマグルチニン刺激ニワトリ末梢血リンパ球の *in vitro* の活性化パターンには同期しなかった。従って，マクロファージの抗原提示はTリンパ球活性化リズムの形成にはあまり寄与しておらず，Tリンパ球の活性化のリズムは他の要因あるいはT細胞活性の固有の変化によるものと思われる。

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キーワード：日内リズム，ニワトリマクロファージ，Fcレセプター，スーパーオキサイド，抗原提示