Ovarian autoimmunity in relation to egg production in laying hens

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The aim of this study was to determine whether anti-ovarian autoantibodies appear in the circulation of laying hens and whether the concentrations of these antibodies change with respect to ageing and egg laying rate. Autoantibodies to ovarian tissues in the circulation of aged (aged approximately 670 days) White Leghorn hens with low (< 50%) and high (> 90%) egg laying rates were examined by ELISA and western blotting. Young laying hens (aged 185 days) with > 95% egg production were used as controls. The results of the ELISA indicated that IgG, which bound to the ovary and small white follicles, was present in the circulation of old laying hens. More hens that laid few eggs had circulatory autoantibodies to the ovary and small white follicles, as determined by the cut-off value in ELISA

(mean absorbance + 2 SD of young laying hens), than did hens that laid greater numbers of eggs, and the concentration of IgG was significantly higher in the hens that laid few eggs. In contrast, when the muscle proteins were used as antigens there were no significant differences in the absorbance values among low and high laying frequency old hens or young hens. Western blotting revealed many bands of immunoprecipitates formed by ovarian antigens and antibodies in the serum of old hens, indicating the presence of many binding sites for circulatory IgG in ovarian tissues. These results indicate that antibodies to ovarian tissues appear in the circulation of laying hens during ageing, and that the concentration of these autoantibodies is related inversely to the rate of egg laying by hens.

Introduction

In hens, egg production decreases with the increase in follicular atresia that occurs during ageing (Bahr and Palmer, 1989). Waddington *et al.* (1985) reported that atresia of small follicles occurred at a higher incidence in old hens than young hens. Endocrinological studies have demonstrated that lack of adequate gonadotrophin support may be one of the major factors for the increased incidence of atresia with age in birds (Bahr and Palmer, 1989) and mammals (Richards, 1980). Johnson (1996) reported that follicular atresia in hens was mediated via a specific type of programmed cell death – apoptosis. This author also proposed that humoral factors and intracellular mechanisms might promote follicular atresia in pre-hierarchical follicles.

However, ovarian disorders in mammalian species also occur as a result of ovarian autoimmunity (Coulam, 1982; Kim *et al.*, 1995). Irvine *et al.* (1968) reported that circulating humoral autoantibodies in women are a contributory factor to follicular death. Autoimmunity is a condition in which an immune response is directed against self tissues or components and may develop pathological consequences leading to disease (Benjamini and Leskowitz, 1991). In ovarian autoimmunity, immune responses occur

against self ovarian tissues, resulting in ovarian disorders and follicular depletion (Coulam and Stern, 1991). Oocytes of the developing follicles, the zona pellucida, theca and granulosa cells are antigenic ovarian structures in mammalian ovaries (Cameron *et al.*, 1988; LaBarbera *et al.*, 1988; Monacayo and Monacayo, 1992). Vallotton and Forbes (1966) reported that antibodies against the ooplasm were present in the circulation of women with ovulation failure. Circulatory autoantibodies to gonadotrophin receptors may block gonadotrophin action, resulting in follicular atresia and ovarian dysfunction in mammals (Ruehsen *et al.*, 1972; Austin *et al.*, 1979; Chiazuzzi *et al.*, 1982; Kim *et al.*, 1995).

Coulam and Stern (1991) proposed that abnormal expression of major histocompatibility complex class II (MHC II) antigens on the ovarian tissues and decreases in ovarian immunity might be the primary factors for induction of ovarian autoimmunity in mammals. In contrast, there is no information about whether ovarian autoimmunity occurs in hens. If autoantibodies appear in hens they may enhance atresia and, thus, reduce egg production. MHC II expressing cells, T cell subsets and immunoglobulin-containing cells are present in hen ovaries and their populations decrease during ageing of hens (Barua *et al.*, 1998a,b; Barua and Yoshimura, 1999a,b). Therefore, it is possible that decreases and abnormalities in ovarian immunity may lead to induction of ovarian autoimmunity in aged hens, as has been proposed for mammals. The aim of the present study was to determine

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whether anti-ovarian antibodies appear in the circulation of laying hens and whether the concentrations of these antibodies change with respect to ageing and egg laying rate.

Materials and Methods

Birds

Young White Leghorn laying hens (aged 185 days with > 95% egg production; $n = 10$) and old laying hens (aged approximately 670 days) were kept in individual cages under a light regimen of 14 h light:10 h dark with food and water available *ad libitum*. The egg laying rates of the young hens were monitored approximately 15 days before the experiment. The laying patterns of old hens (experimental birds) were monitored for 2 months and the hens were then divided into two groups: hens with < 50% egg production (low laying frequency old hens; $n = 10$) and hens with > 90% egg production (high laying frequency old hens; $n = 10$). Serum samples were obtained from all birds and kept at -20°C until used. Serum samples from young laying hens were used as control serum in ELISA.

Preparation of antigens

Ovarian tissues, small white follicles (3–4 mm in diameter) and skeletal muscle were collected from normally laying hens (aged approximately 210 days) of the experimental group and used as the antigens for ELISA and western blotting. The samples were homogenized with homogenization buffer (1% (v/v) NP-40; 0.1% (w/v) sodium deoxycholate; 1% (v/v) PMSF, 150 mol NaCl l^{-1} Tris-HCl; pH 7.5) using a Polytron homogenizer (PT 1200; Kinematica AG, Switzerland) on ice. Homogenized tissue samples were centrifuged for 15 min at 6200 g (MRX-150; Tomy Seiko Co, Tokyo) and the supernatants were collected. The protein concentrations in each sample were determined using protein assay reagent (Bio-Rad, Hercules, CA) according to the manufacturer's instructions (Bradford, 1976). Samples were kept frozen (-80°C) until used as antigens in ELISA and western blotting.

ELISA

The presence of circulatory autoantibodies was examined by ELISA as described by Wheatcroft *et al.* (1994), with minor modifications. The wells of microtitre plates were coated with antigens by incubating with 100 μl isolated ovarian, small white follicle or muscle protein samples (protein concentration was adjusted to 1 mg ml^{-1} with PBS) for 2 h at room temperature. After antigen coating, excess antigen solution was removed and non-specific sites were blocked with 1% (w/v) casein milk in PBS at 4°C overnight. Wells were incubated with the serum (1:10 dilution in 1% (w/v) BSA-PBS) of experimental birds for 2 h at room temperature. After washing with PBS containing 0.05% (v/v) Tween-20 (3 $\times$ 5 min washes), the wells were incubated with alkaline phosphatase conjugated rabbit anti-chicken IgG (Sigma, St Louis, MO) at a dilution

of 1:30 000 in 1% (w/v) BSA-PBS at 37°C for 2 h. Immunoreactions were developed using a *p*-nitrophenylphosphate substrate solution kit (KPL, Gaithersburg, MD). The plates were read in a microplate reader (Model 550, Bio-Rad) at 405 nm. All samples were assayed in duplicate within the same plate. A background absorbance value was obtained from the control well with the omission of serum. This measurement provided background values that could be subtracted from the absorbance values of experimental samples. After subtraction, the mean of two absorbance values for each sample was calculated and plotted in a dot diagram. Serum of young laying hens ($n = 10$) was used as control serum and the mean absorbance + 2 SD of the control samples was used as a cut-off value between positive and negative for auto-antibodies as described by Luborsky *et al.* (1990) and Wheatcroft *et al.* (1994). The absorbance values greater than the cut-off value were considered as positive for auto-antibodies. The chi-squared test was used for statistical analysis of the frequency of birds with autoimmune serum. The level of significance was $P < 0.01$.

Western blotting

Ovarian or small white follicle antigen samples (1 mg ml^{-1}) were separated by electrophoresis on discontinuous SDS-polyacrylamide gels (4% upper gel and 7.5% lower gel) as described by Laemmli (1970). After electrophoresis the separated proteins in the lower gel were transferred electrophoretically onto nitrocellulose membrane (Hybond-C; Amersham International plc, Amersham) using a semi-dry blotting system with transfer buffer (0.025 mol Tris-glycine l^{-1} (pH 8.3) and 20% (v/v) methanol). The membrane was washed with western buffer (0.15 mol NaCl, 0.5% (v/v) Tween-20 and 0.05% (w/v) BSA in 0.02 mol Tris-HCl l^{-1} ; pH 7.4) for 30 min (3 $\times$ 10 min washes). The membrane was blocked for 1 h at room temperature with 1% (w/v) casein milk in PBS. After blocking, the membrane was cut into antigen-bearing strips. Each strip was incubated with a serum sample (diluted in PBS at a concentration of 1:100 for 2 h at room temperature) from a low or high laying frequency old hen. After washing with western buffer for 30 min (3 $\times$ 10 min washes), the membrane strips were incubated with alkaline phosphatase-conjugated rabbit anti-chicken IgG (Sigma) that had been diluted to 1:5000 in western buffer at room temperature for 2 h. After washing with western buffer (3 $\times$ 10 min washes), the immunoprecipitates on the strips were visualized using a mixture of 3 mg nitroblue tetrazolium and 3 mg 5-bromo-4-chloro-3 indonyl phosphate in 10 ml substrate buffer (0.1 mol Tris-HCl l^{-1} , pH 9.6, containing 1 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$). A background control strip, for which the incubation with serum sample was not performed, was included in each assay to identify the background band signals and endogenous IgG originating from the antigen samples of ovary and small white follicles. The number of immunopositive protein bands on the strips was analysed

Fig. 1. ELISA dot plot diagram for anti-ovarian antibodies in hen serum samples. Binding of IgG to proteins isolated from the ovaries of hens was examined in the serum of low laying frequency old hens (aged approximately 670 days with < 50% egg production), high laying frequency old hens (aged approximately 670 days with > 90% egg production) and young hens (aged 185 days with > 95% egg production) ($n = 10$ birds in each group). The dotted line shows the cut-off value, which is the mean absorbance + 2 SD of control (young laying hen serum).

by a computer assisted software program (Gel-Pro Analyzer; Media Cybernetics, The Image Experts, ML).

Results

ELISA for anti-ovarian and anti-small white follicle antibodies

The ELISA absorbance of serum from experimental (low or high laying frequency old hens) and control (young laying hens) birds against the ovary, small white follicle and skeletal muscle antigens are shown (Figs 1, 2 and 3, respectively). All the absorbance values of serum from low laying frequency old hens against the ovary and small white follicle antigens were above the cut-off value (mean absorbance + 2 SD of control). Only two of ten serum samples from high laying frequency old hens had an absorbance value greater than the cut-off line in the analysis of both ovary and small white follicle antigens. In the young laying hen group none of the serum samples had an absorbance value greater than the cut-off line against the ovary and the small white follicle antigens. The frequency of birds with absorbance values above the cut-off line for both the anti-ovary and anti-small white follicle antibodies was significantly higher for low laying frequency old hens than high laying frequency old hens ($P < 0.01$). The correlation between the individual absorbance values and egg laying rates was not significant within the low laying frequency old hen group. However, the mean absorbance values in low laying frequency old hens (ovary:

Fig. 2. ELISA dot plot diagram for anti-small white follicle antibodies in hen serum samples. Binding to proteins isolated from small white follicles was analysed in the serum of low laying frequency old hens (aged approximately 670 days with < 50% egg production), high laying frequency old hens (aged approximately 670 days with > 90% egg production) and young hens (aged 185 days with > 95% egg production) ($n = 10$ birds in each group). The dotted line shows the cut-off value, which is the mean absorbance + 2 SD of control (young laying hen serum).

1.19 ± 0.05 ; small white follicle: 73 ± 0.11) were significantly higher than in either high laying frequency old hens (ovary: 0.87 ± 0.04 ; small white follicle: 21.43 ± 0.03) or control hens (ovary: 0.73 ± 0.04 ; small white follicle: 1.16 ± 0.06) in the analysis of both the ovary and small white follicles ($n = 10$; $P < 0.01$). In contrast, no absorbance value was found to be above the cut-off value in all serum samples from both experimental and control birds for muscle antigens.

Western blotting for anti-ovarian and anti-small white follicle antibodies

The results of immunoblotting for the serum from low laying frequency or high laying frequency old hens against the ovary and small white follicles are shown (Figs 4 and 5). In both tissues, many bands of various sizes were observed in the serum of both low and high laying frequency old hens. Although one of four birds in the high laying frequency old hen group showed some dense bands in the ovarian antigen, the number of bands was greater in samples from low laying frequency old hens than in samples from high laying frequency old hens.

Discussion

This is the first report of the appearance of ovarian autoimmunity in laying hens. The significant findings of this experiment are: (i) IgG, which binds to the ovarian tissues, was present in the circulation of old laying hens; (ii) the frequency of birds with higher concentrations of this IgG was significantly greater in the low laying frequency old hen group than in the high laying frequency old hen

Fig. 3. ELISA dot plot diagram for anti-muscle antibodies in hen serum samples. Binding of IgG to skeletal muscle proteins was examined in the serum of low laying frequency old hens (aged approximately 670 days with < 50% egg production), high laying frequency old hens (aged approximately 670 days with > 90% egg production) and young hens (aged 185 days with > 95% egg production) ($n = 10$ birds in each group). The dotted line shows the cut-off value, which is the mean absorbance + 2 SD of control (young laying hen serum).

group; and (iii) the serum IgG from old hens bound to molecules of various sizes in ovarian and small white follicle samples.

In ELISA analysis, the mean absorbance + 2 SD of control has been proposed as the cut-off value between negative and positive sera for detection of anti-ovarian antibodies in mammals (Luborsky *et al.*, 1990; Wheatcroft *et al.*, 1994; Fenichel *et al.*, 1997). These authors proposed that absorbance values greater than the cut-off value are indicative of the presence of autoantibodies. In the present study, all of the serum samples from low laying frequency old hens had absorbance values greater than the cut-off value, which is indicative of the presence of autoantibodies to the ovary and small white follicles. In western blotting of ovarian and small white follicle peptides, many bands were observed in all the serum samples from both low and high laying frequency old hens. This finding indicates that there are antigenic molecules of various sizes for the circulating autoantibodies in these tissues. Similar findings were also reported by Wheatcroft *et al.* (1994), who observed many immunoreactive bands of various sizes using serum from women with autoimmune disease against ovarian antigens. In contrast, binding of the serum of old laying hens to skeletal muscle peptides was not observed. Therefore, it is likely that autoimmunity in low laying frequency old hens occurred against tissue-specific proteins; the ovary is a tissue that contains such proteins.

The frequency of hens with ovarian autoantibodies

Fig. 4. Western blotting for serum from old laying hens against ovarian antigens. Low laying frequency: hens aged approximately 670 days with < 50% egg production (individual hens 1–4). High laying frequency: hens aged approximately 670 days with > 90% egg production (individual hens 1–4). C: background control in which serum was omitted. Values in the left margin indicate the molecular size of protein bands. Dense bands of approximately 40–48 kDa show endogenous IgG that was contained in the ovarian tissue as they appear in lane C.

(above the cut-off value) was significantly greater in low laying frequency old hens than in high laying frequency old hens. Although the mean amount of autoantibody in older hens was not determined in the present study, the absorbance values showing the concentration of antibodies to ovarian tissues were significantly greater in the serum of low laying frequency old hens than in high laying frequency old hens. Therefore, the current findings indicate that there may be a correlation between decreased egg production rate and increased ovarian autoantibody concentrations.

Although the mechanism by which ovarian autoimmunity occurs is not understood fully, several possibilities have been suggested for mammalian ovaries. Environmental insults, such as noxious chemicals, industrial waste, viruses and other pathogens may invade ovarian tissues (Hill *et al.*, 1990). As a consequence of a depleted population of CD8 T cells during ageing, environmental agents or viral infection may induce interferon (IFN)- α or - β .

Fig. 5. Western blotting for serum from old laying hens against small white follicle antigens. Low laying frequency: hens aged approximately 670 days with < 50% egg production (individual hens 1–4). High laying frequency: hens aged approximately 670 days with > 90% egg production (individual hens 1–4). C: back-ground control in which serum was omitted. Values in the left margin indicate the molecular size of protein bands. Dense bands of approximately 40–48 kDa show endogenous IgG that was contained in the ovarian tissue as they appear in lane C.

These cytokines can activate CD4 T cells to produce IFN- γ . Increased production of IFN- γ may induce abnormal expression of MHC II antigens in ovarian tissues, as well as other organs, triggering the synthesis of autoantibodies in women (Bottazzo *et al.*, 1983; Hill *et al.*, 1990; Coulam and Stern, 1991). Michael (1983) reported that a balanced ratio of CD4:CD8 T cells was essential for normal ovarian function and an increased CD4:CD8 T cell ratio could stimulate the production of ovarian autoantibodies in mammals. In birds, autoimmunity may occur against the thyroid gland and a possible mechanism of induction has been proposed (Witebsky *et al.*, 1969; Katz *et al.*, 1981). Macrophages can induce inappropriate responses against chicken tissues and may cause localized tissue destruction (Klasing, 1991). For example, activated macrophages are implicated in mediating spontaneous autoimmune thyroiditis in obese chicken lines, possibly due to impaired CD8 T cell activity (Diertert *et al.*, 1987). From the studies of autoimmunity in mammalian ovaries and avian thyroid glands, the common features in induction of autoimmunity

are likely to be immunity decline, disruption in MHC II expression to recognize self by macrophages or other antigen presenting cells and imbalance in CD4 and CD8 T cell populations.

Recently, the presence of macrophages (Barua *et al.*, 1998a), MHC II expressing cells (Barua and Yoshimura, 1999a) and T cell subsets (Barua and Yoshimura, 1999b) in hen ovaries have been reported. Although it is not known whether functional disorder of MHC-II occurs in hen ovaries, the populations of MHC-II expressing cells, macrophages and T cell subsets decreased with ageing, indicating that there is a decrease in immunity during ageing (Barua *et al.*, 1998a; Barua and Yoshimura, 1999a,b). Furthermore, CD4:CD8 ratio was increased in old hens with a greater decrease in CD8 T cell populations than in CD4 T cell populations (Barua and Yoshimura, 1999b). These findings indicate that the population of immunocompetent cells in hen ovaries is likely to change during ageing in a similar way to autoimmune induction in mammalian ovaries and avian thyroid glands. Therefore, during ageing, hen ovarian autoimmunity may be induced by disordered ovarian immune function, including decreased immunity and possibly self-recognition by MHC-II and abnormal T cell function.

In conclusion, the results of the present study indicate that anti-ovarian antibodies appear in the circulation of laying hens during ageing. The concentration of anti-ovarian antibodies was significantly higher in low laying frequency old hens than in high laying frequency old hens, indicating that there is an inverse correlation between the appearance of anti-ovarian antibodies and egg production. It is possible that ovarian autoantibodies are one factor responsible for decreased egg production by old hens.

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