The influence of ovarian hormones on the granulomatous inflammatory process in the rat lung

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The influence of ovarian hormones on the granulomatous inflammatory process in the rat lung. M. Shirai, A. Sato, K. Chida. ©ERS Journals Ltd 1995.

ABSTRACT: This study was initiated to clarify the relationship between ovarian hormones and the granulomatous inflammatory process in the lung.

To assess whether ovarian dysfunction influences the granulomatous inflammatory process, we compared immunological alterations in ovariectomized rats and in sham-operated rats. After a heat-killed, bacilli Calmette-Guérin (BCG)-elicited granulomatous reaction, the lung-body weight ratios, the number of lymphocytes and activated T-cells, and the interferon-gamma levels in the bronchoalveolar lavage fluid from the ovariectomized rats were significantly higher than those of the sham-operated rats. Moreover, exogenous ovarian steroids supplemented *in vivo* suppressed not only the granulomatous inflammatory process in the lungs, but also the parameters measured in the bronchoalveolar fluid.

These results indicate that ovarian dysfunction may adversely affect the formation of granulomas in the lung.

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The interaction between the reproductive organs and the immune system has become a subject of increased focus, and gonadectomy, a sex hormone replacement, or pregnancy are all known to cause an altered immune response, suggesting the presence of a specific receptor for the gonadal steroids in the immune organs [1]. However, few reports have appeared concerning the influence of hormones on the formation of granulomas in the lung [2, 3]. Most cases of sarcoidosis in female patients occur between 20 and 30 yrs of age, and after 40 yrs of age [4–6]. Furthermore, we have found that the resolution of this disease in Japanese female patients is less likely after menopause, and that childbirth has an effect on sarcoidosis activity (paper submitted). It has been reported that sarcoidosis improves during pregnancy [7].

Although the above findings suggest that the patient's endocrinological status may be related to sarcoidosis activity, what still remains unclear is how the mechanisms of granuloma formation are associated with the patient's hormonal status. This study was, thus, initiated to ascertain whether an ovariectomy affects the granulomatous inflammatory process in the rat lung, and which of the hormones severely affect the formation of granulomas.

Materials and methods

The rat treatment regimen (fig. 1)

For this study, we used specific, pathogen-free, female, 10 week old, DA rats, weighing 150–200 g, purchased

from Shizuoka Agricultural Co-operative Association for Laboratory Animals (Shizuoka, Japan). After housing these rats under normal day/night cycles, both ovaries of 32 rats were removed, under ether anaesthetization, through an incision in the back. The decrease in the serum oestradiol and progesterone levels in these ovariectomized (OV) rats was subsequently evaluated. The concentrations of serum oestradiol and progesterone in OV rats and SHAM rats were as follows; 1) oestradiol of SHAM (n=5) 20.9±5.0 pg·ml⁻¹; 2) oestradiol of OV (n=5) not detectable; 3) progesterone of SHAM (n=5) 11.3±8.3 ng·ml⁻¹; and 4) progesterone of OV (n=5) 9.5±3.1 ng·ml⁻¹.

Four weeks later, after the hormonal status of these OV rats had stabilized, we compared the data in the OV rats (n=5) and SHAM rats (n=5) before administration of heat-killed bacillus Calmette-Guérin (BCG) (gift from Q.N. Myrvik). Then the OV rats were sensitized with intravenous injection of 50 µg heat-killed BCG, after which this rat group was referred to as the OV+BCG rats. Twenty one BCG-injected rats were given a sham operation (SHAM+BCG) and served as controls.

To ascertain the effect of supplemented sex hormones, from Day 15–21, three sets of OV+BCG rats received a subcutaneous injection of one or more of the following hormones: 0.1 mg·day⁻¹ β-oestradiol 3-benzoate (E2; Sigma Chemical Co., St. Louis, MO, USA); or 0.5 mg·day⁻¹ progesterone (Pr; Sigma); or an injection of both E2 and Pr. The three sets of rats were then, respectively, referred to as the OV+BCG+E2 group (n=10), the OV+BCG+Pr group (n=7), and the OV+BCG+E2+Pr group (n=5). Prior to these injections, each hormone

Fig. 1. — Diagram of the rat treatment regimens. I: Groups without heat-killed BCG; II: Groups with heat-killed BCG. Arrows indicate sensitization with a 50 µg intravenous injection of heat-killed BCG. OV: ovariectomy; SHAM: sham operation; E2: oestradiol; Pr: progesterone; BCG: bacilli Calmette-Guérin; sc: subcutaneous.

was dissolved in propylene glycol, since the use of this medium for administering the injection does not provoke an inflammatory response.

Cell preparation

Under anaesthesia, each rat was exsanguinated through the descending aorta. After aseptic removal of both lungs, the lung weight of each rat was measured. This was followed by bronchoalveolar lavage (BAL) of each rat via a polyethylene catheter introduced into the trachea. BAL was performed by instilling 0.9% NaCl in three 5 ml aliquots. Following lavage and the cyto-centrifugation of each BAL sample, the cell pellets that resulted were washed twice in phosphate buffered saline (PBS) (pH 7.4). The total cell count of each pellet and cellular

differentiations were ascertained by routine haemocytometry and by May-Grünwald-Giemsa staining, respectively. The cells were then suspended in PBS at a final concentration of 1.0×10^6 cells·ml⁻¹.

Histology

A tissue specimen taken from each lung was fixed in a 10% formaldehyde solution and embedded in paraffin for haematoxylin-eosin staining.

Flow cytometric analysis

The surface expression of the T-cell subsets in the BAL cells was calculated by indirect immunofluorescence, using monoclonal antibodies (MoAbs). W3/25 (Serotec, Oxford, UK; CD4), raised against rat T-helper cells, and OX8 (Serotec, Oxford, UK; CD8), raised against rat T-nonhelper cells were used to identify the T-cell subsets. The BAL cells, prepared as described previously, were preincubated with heat-inactivated rat serum for 30 min on ice, after which they were incubated for 60 min at 4°C with the MoAbs, W3/25 or OX8 as the first antibody. After three washings, the BAL cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat F(ab')₂ anti-mouse immunoglobulin G (IgG) (TAGO, Inc., Burlingame, CA, USA) as the second antibody. Flow cytometric analysis was performed with an Epics-Profile (Coulter Electronics Inc., Hialeah, FL, USA).

To examine the activated, Ia antigen positive T-cell population, a double immunofluorescence analysis was employed. In brief, both a W3/13 fluorescein conjugate and a OX6 phycoerythrin conjugate, raised against rat pan T-cells and Ia antigens, respectively, (Serotec, Oxford, UK), were incubated with BAL cells, 1.0×10^6 cells·ml⁻¹, and then analysed.

Determination of interferon-gamma in BAL fluid

The interferon-gamma (IFN-γ) value in the BAL fluid of each group was determined by means of an enzyme-linked immunosorbent assay (ELISA) (HBT rat interferon-gamma ELISA Testkit, Holland Biotechnology). Prior to this ELISA, the BAL fluid samples were concentrated up to 50 times by Minicon-B (Amicon Division, W.R. Grace & Co., Conn, USA). The IFN-γ values were then corrected, according to the total protein concentration in the BAL fluid, which was measured by means of a colorimetric assay (DC protein assay kit, BIO-RAD, Richmond, CA, USA).

Statistical analysis

The results of all data are shown as mean ± standard deviation. A single factor analysis of variance (ANOVA) and Bonferroni's method were used to compare differences between each group, as shown in figure 1. A p-value of less than 0.05 was considered to be statistically significant.

Fig. 2. – Histological findings in a sample rat lung specimen from each group. a) non-operated rat; b) OV+BCG rat with inset panel showing higher magnification ($\times 50$); c) SHAM+BCG rat; d) OV+BCG+E2 rat; e) OV+BCG+Pr rat; f) OV+BCG+E2+Pr rat. For abbreviations see legend to figure 1. (Haematoxylin and eosin (HE) staining; magnification $\times 25$; internal scale bar=200 μm).

Results

Before administration of heat-killed BCG, the OV and SHAM rats were no different with respect to lung/body weight ratios and BAL findings, *i.e.* the total cell count, lymphocyte counts, activated T-cells, and IFN- γ levels. Similarly, no differences in these parameters were found between the SHAM rats and the SHAM rats injected with E2 and/or Pr (data not shown).

The histological findings of the lung tissue specimens were compared among the groups (fig. 2). In the OV+BCG group (fig. 2b), diffuse, mature, epithelioid-cell granulomas can be seen, whereas in the lung tissue specimens from the SHAM+BCG group, epithelioid-cell granulomas were sparse and less well-developed (fig. 2c). Further, in the groups supplemented with injections of E2 and/or Pr (figs. 2d–f) the reactions were suppressed in comparison to the OV+BCG group.

Figure 3 shows the lung/body weight ratios of the different groups, reflecting the degree of the inflammatory

Fig. 3. – The distribution of lung/body weight ratios. The lung/body weight ratios of the OV+BCG rats were significantly higher than those of the SHAM+BCG rats (OV+BCG vs SHAM+BCG 14.4 ± 5.7 vs 9.2 ± 1.3 ; $p < 0.05$). Values are presented as mean $\pm$ SD. *: $p < 0.05$, compared to the OV+BCG value. For abbreviations see legend to figure 1.

Table 1. – Cell populations of bronchoalveolar lavage

	Total $\times 10^5 \cdot \text{ml}^{-1}$	%		
		Mac	Lymph	PMN
OV	0.52 $\pm$ 0.1	98.4 $\pm$ 0.9	1.6 $\pm$ 0.9	0
SHAM	0.51 $\pm$ 0.1	99.5 $\pm$ 0.3	0.4 $\pm$ 0.3	0.06 $\pm$ 0.1
OV+BCG	2.24 $\pm$ 1.28	77.7 $\pm$ 12.4	14.9 $\pm$ 12.8	7.4 $\pm$ 6.2
SHAM+BCG	1.64 $\pm$ 0.73	87.3 $\pm$ 5.7*	8.0 $\pm$ 5.4*	2.7 $\pm$ 3.6*
OV+BCG+E2	1.18 $\pm$ 0.40*	95.9 $\pm$ 2.5*	3.7 $\pm$ 1.8*	0.7 $\pm$ 1.4*
OV+BCG+Pr	0.94 $\pm$ 0.63*	99.1 $\pm$ 0.8*	0.8 $\pm$ 0.8*	0.07 $\pm$ 0.2*
OV+BCG+E2+Pr	1.34 $\pm$ 0.20	92.4 $\pm$ 4.7*	5.4 $\pm$ 2.7	2.2 $\pm$ 2.2

Data are presented as mean $\pm$ SD. MAC: macrophage; Lymph: lymphocyte; PMN: polymorphonuclear leucocyte; OV: ovariectomy; BCG: bacilli Calmette-Guérin; SHAM: sham operation; E2: oestradiol; Pr: progesterone. *: $p < 0.05$ compared to the OV+BCG value.

Fig. 4. – Lymphocyte subsets in the bronchoalveolar lavage (BAL) fluid. a) the number of W3/25+ (CD4+) cells in the BAL fluid. b) the number of OX8+ (CD8+) cells in the BAL fluid. The number of W3/25+ (CD4+) cells and OX8 (CD8+) cells in the OV+BCG group was higher than that in the SHAM+BCG or E2 group. Values are presented as mean $\pm$ SD. *: $p < 0.05$ compared to the OV+BCG value. ND: nondetectable. For further abbreviations see legend to figure 1.

Fig. 5. – Activated T-cells in the bronchoalveolar lavage (BAL) fluid. a) the number and b) the percentage of activated T-cells in the BAL fluid. There was a significantly higher number and percentage of activated T-cells in the OV+BCG group, suggesting that ovarian dysfunction is associated with T-cell activation. Values are presented as mean $\pm$ SD. *: $p < 0.05$, compared to the OV+BCG. For abbreviations see legend to figure 1.

Fig. 6. – Interferon-gamma (IFN- γ) content in bronchoalveolar lavage (BAL) fluid. For abbreviations see legend to figure 1.

process. The ratios in the OV+BCG group were significantly higher than in the SHAM+BCG group. However, the supplementary injection of E2 and/or Pr helped the OV+BCG group to maintain the same ratio level as the SHAM+BCG group.

As shown in table 1, the total BAL cell count in the OV+BCG group was highest. The total cells in BAL fluid from the E2 or Pr+BCG groups showed a significant decrease in comparison to the OV+BCG group. With respect to the cell differentiations of each group, the OV+BCG group had significantly higher lymphocyte and PMN values and, conversely, the percentage of macrophages in the OV+BCG group was significantly lower than in the other groups.

With respect to the T-lymphocyte subsets in the BAL fluid, the CD4 (W3/25)-positive T-cells in the OV+BCG group outnumbered those of the SHAM+BCG or OV+BCG+E2 groups (fig. 4), although no statistically significant differences were seen between the OV+BCG and SHAM+BCG groups. The CD4+ and CD8+ (OX8) cells in the OV+BCG+Pr and OV+BCG+E2+Pr groups could not be determined by flow cytometry, because too few lymphocytes were recovered. In the OV+BCG, SHAM+BCG and OV+BCG+E2 groups, CD4+ T-cells predominated over the CD8+ T-cells. Figure 5 shows T-cells coexpressing W3/13 and OX6, which are considered to be activated T-cells. Although the activated T-cells varied with regard to percentage among the groups, the total number of activated T-cells in the OV+BCG group was significantly higher than in the other groups. The IFN- γ levels in the BAL fluid were significantly elevated in the OV+BCG group, $p < 0.05$ (fig. 6).

Discussion

According to several investigators [4–6], in older women

with sarcoidosis, a second peak of sarcoidosis incidence occurs between the ages of 45 and 65 yrs. In addition, we have previously reported that the resolution of sarcoidosis is less likely in Japanese females after menopause (paper submitted). However, as sarcoidosis is thought to be closely associated with T-cell activation, these findings appear to contradict the finding that T-cell activity declines with age [8, 9]. This has led us to suspect that hormonal changes may affect sarcoidosis activity. To explore this possibility more fully, we investigated the relationship between alterations in ovarian function and the granulomatous reaction. Although much effort has been spent in trying to determine the relationship between hormonal activity and immunity, reports on ovarian dysfunction and immunity are few [10–12], and no previous report has focused on the influence of the sex steroids on pulmonary immunity.

The results of our study on ovariectomized rats support the speculation that ovarian dysfunction enhances the granulomatous inflammatory process in the rat lung, based on comparing this same process in sham-operated rats. In another rat study, CHRISTOPHER and ISTVAN [11] reported that an ovariectomy resulted in a significant increase in the mitogen response of the peripheral lymphocytes, compared to the response in sham-operated rats. JILKA *et al.* [12] found that the levels of interleukin-6 (IL-6) in ovariectomized mice was significantly higher than in sham-operated mice.

The focus of this study has been to determine which ovarian hormone is mainly responsible for enhancing the process of granuloma formation in ovariectomized rats. We thus investigated whether β -oestradiol 3-benzoate and/or progesterone enhances this granulomatous process in the rat lung, and found that both hormones suppressed the process in the ovariectomized rat, leading us to speculate that a lack of ovarian hormones may be responsible for augmenting the granulomatous inflammatory process.

Several reports support this speculation. CHRISTOPHER and ISTVAN [11] found that injections of oestradiol valerate suppressed the mitogenic response of the peripheral blood lymphocytes of ovariectomized rats. Based on a study of mice, JILKA *et al.* [12] reported that 17β -oestradiol suppressed IL-6 production by the stromal cells of the bone marrow and increased the number of colony-forming units of granulocytes and macrophages in ovariectomized mice. Additionally, in a study of postmenopausal women, MANYONDA *et al.* [13] found that 17β -oestradiol suppressed the delayed cutaneous hypersensitivity response and the mixed lymphocyte reaction. However, as a contrary finding, oestradiol has been reported to increase the activity of the IFN- γ promoter in mice lymphoid cells [14]. With regard to the effect of the sex hormones on lymphoid tissue and the cytokines, the interactions are quite complicated and remain poorly understood [1, 15].

Concerning the influences of an ovariectomy on the mechanisms of granuloma formation, this rat study found that the altered endocrinological environment caused by an ovariectomy activated BAL T-cells and provoked a subsequent IFN- γ production. Our results, thus, indicate that ovarian dysfunction may adversely affect the formation of granulomas in the lung.

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