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Murine lymphokines production in lymphoid organs during the various stages of lactation

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비유기간에 따라 murine 림프조직에서 생산되는 lymphokine의 양상에 관한 연구

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초 록 : 비유중기, 건유초기 및 임신말기의 C3H/HeN 마우스의 비장, 장간막림프절, 말초림프절(액와, 상완 및 샅림프절) 유래 림프구에서 생산되는 lymphokine의 양을 비교하였다. 비장에서의 IL-2 생산양은 임신말기와 비유중기에 낮았지만, IL-4, IL-6와 IFNγ의 생산양은 임신말기와 건유초기에 높았다. 말초림프절에서의 IL-4, IL-6 및 IFNγ 생산양은 임신말기와 건유초기에 높았지만, IL-2의 생산양은 임신말기, 비유중기 및 건유초기에 각각 감소하였다. 장간막림프절에서의 IL-4 생산양은 임신말기와 비유중기에 각각 증가하였으나, IL-2의 생산양은 임신말기에 감소하였다. 이와같이 전반적으로 건유초기와 임신말기에 IL-2의 생산양은 낮지만, IL-4, IL-6와 IFNγ의 생산양이 높은 결과는 이 시기에 유방염의 발생비율이 높은 것과 연관성이 있는 것으로 추정된다.

Key words : Lymphokine, lactation, mammary gland involution, pregnancy

Introduction

Many published studies on intramammary infections(IMI) have established that the risk of IMI is greatest during the first 2 weeks of mammary gland involution and the 2 weeks preceding parturition[1-4]. Presently, numerous studies have demonstrated that the composition of lymphocyte subpopulations vary during the lactation cycle[5-7]. However, little information is available concerning the mechanisms responsible for causing these changes during cycles of lactation.

It is believed that steroid hormones are in some way responsible for altering the pattern of cytokines produced in response to an inflammatory or immunologic challange. This concept is supported by studies which demonstrate that enriched populations of T cells from normal donors, as well as cloned T-cell lines(capable of both interleukin(IL)-2 and IL-4 production following activation) secrete reduced levels of IL-2 and interferon γ(IFNγ), and elevated levels of IL-4 subsequent to a direct exposure to physiologic

or pharmacologic levels of glucocorticoids *in vitro or in vivo*[8,9].

Changes in the production of steroid hormones capable of affecting mammary gland function and their association with the various stages of lactation have been well described in animals[10]. Therefore, it is not unreasonable to suggest that the capacity of a host to produce the various types of lymphokines and cytokines may be susceptible to change by various exogenous influences, both at the cell and organ system levels. The present study has investigated the alterations in inducible lymphokines produced by activated T cells from female donors during the various stages of lactation.

Materials and Methods

Mice : C3H/HeN strain of mice were bred and housed in the University of Utah Vivarium from breeding stock originally purchased from the National Cancer Institute. Mice utilized for this study were between 13-24 weeks in age and the number of female mice in a single experimental group were 8 to 10. Control donors were selected from the different stages of estrous cycle and then evaluated simultaneously. The experimental mice were divided into three groups: pregnancy(1 day prepartum); latation(10 days postpartum); involution(3 days after drying off). The animal facility at the University of Utah guarantees strict compliance with regulations estabilished by the Animal Welfare Act.

Culture conditions : Briefly, single cell suspensions of lymphoid cells were prepared from peripheral lymph nodes(axillary, brachial, inguinal), mesenteric lymph nodes, or spleen, washed twice in sterile balanced salt solution and cultured, serum-free conditions, at a density of 1×10^7 cells/ml/well with a T-cell speicific mitogen, anti-CD3ε, in a 24-well Cluster culture plate(Costar, Cambridge, MA) for a period of 24 hours to elicit lymphokine secretion. The hybridoma clone producing hamster anti-murine CD3ε monoclonal antibody, 1452C-11.2, was obtained from J. Bluestone(University of Chicago). Cell-free culture supernatants were collected and stored at -20℃ until assayed for lymphokine content. The culture period, cell concentrations, and culture medium, consisting of RPMI 1640 supplemented with 1% Nutridoma-SR(Boehringer-Mannheim, IN), antibiotics, 2.0mM L-glutamine and 5×10^{-5} M 2-mercaptoethanol, were all carefully evaluated to determine the optimal conditions for stimulating production of the lymphokines under evalution.

Quantitation of lymphokines

Antibodies : Monoclonal antibody reagents used in the present study were prepared from culture supernatants of B-cell hybridomas adapted to growth under serum-free conditions. The hybridoma clones secreting rat anti-murine IL-2(S4B6) and IFNγ(XMG 1.2) were obtained from DNAX(Palo Alto, CA). The hybridoma clones producing antibody specific for murine IL-4(11B11) and murine IFNγ(R46A2) were purchased from the ATCC. Monoclonal rat anti-murine IL-6 antibodies were purchased from PharMingen(San Diego, CA). Purified rabbit anti-murine IL-2 was purchased from Collaborative Biomedical(A subsidiary of Becton-Dickinson, MA). Purified, biotinylated rat anti-murine IL-4 and IL-6 antibodies were purchased from PharMingen.

Cytokine standards : Murine recombinant IFNγ was obtained from Genentech(5×10^6 units/mg protein) and used as a reference in the IFNγ assays. Murine recombiant IL-2 and IL-4 were derived from culture supernatants of X63Ag8-653 cells transfected with multiple copies of a single murine interleukin gene[11]. After the relative concentration of each lymphokine in culture supernatants was determined by a comparision to a known recombinant standard, these reagents were used as reference lymphokines in capture ELISA. Other sources of purified murine reference lymphokines were IL-6 purchased from PharMingen, or IL-2 and IL-4 purchased from Collaborative Research Inc.(Bedford, MA).

ELISA assay : The amount of cytokine present in a test supernatant was quantitated by a capture ELISA, adapted from the method of Schumacher et al[12]. Briefly, 100μl of 2μg/ml capture antibody in 0.05M Tris-Hcl(pH 9.6) was adsorbed to the wells of a 96-well microtest plate, washed, and blocked with PBS/1%BSA. Test supernatants and 2-fold serial

dilutions of the appropriate reference cytokine(100μl/well) were dispensed, and after sufficient incubation and washing, 100μl of biotinylated-detection antibody, 1μg/ml, was dispensed into each well. The ELISA was developed using avidine-HRP and ABTS-substrate. Spectrophotometric readings were recorded at 405 nM. The limit of detection for most of these cytokines is 15-30pg/ml.

The mean and SEM were calculated and comparisions between a control mice and each group made by student's t-test, with differences between mean values of $p<0.05$ or $p<0.01$ considered significant.

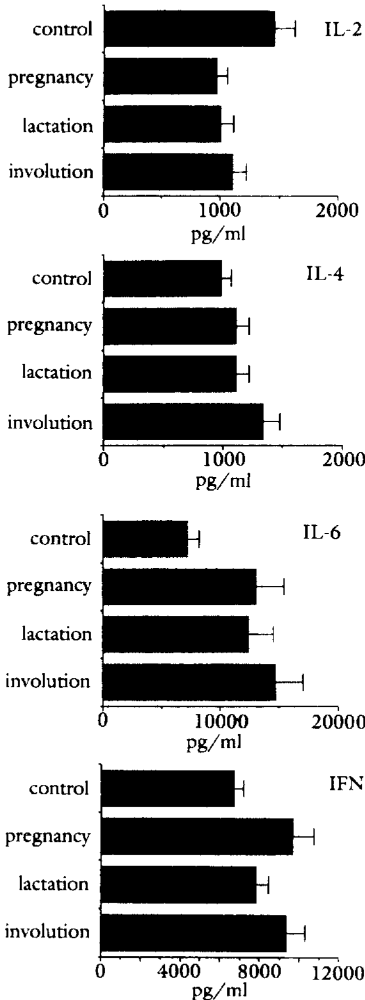

Fig 1. The pattern of lymphokines produced by in vitro activated T cells obtained from the spleens of perilactation mice and normal controls. Data are mean±SEM.

Results

Using a serum-free culture system, lymphocytes from peripheral lymph nodes, spleen, and mesenteric lymph nodes during the various stages of lactation were compared for lymphokine production following T-cell activation with an optimum amount of anti-CD 3ε. After 24 hours of incubation, cell supernatants were analyzed for their lymphokine content according to the procedures described in the Materials and Methods.

Fig 1 provides results which demonstrates that *in vitro* activation of splenocytes from mice which is in

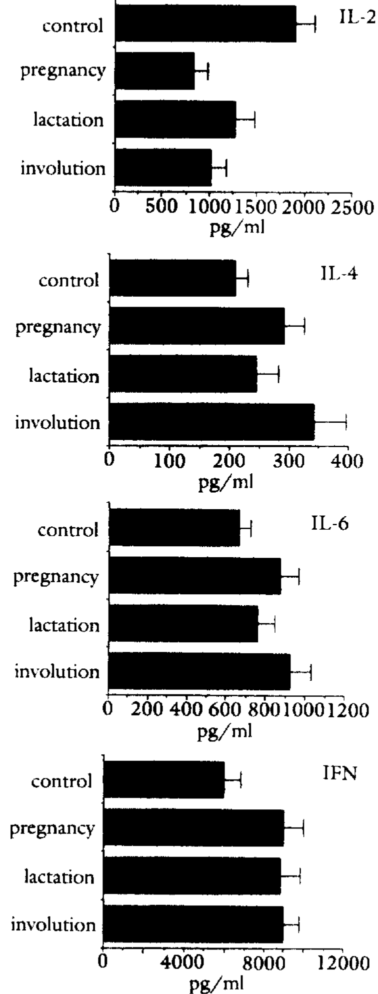

Fig 2. The pattern of lymphokines produced by in vitro activated T cells obtained from the peripheral lymph nodes(axillary, brachial and inguinal) Data are mean±SEM.

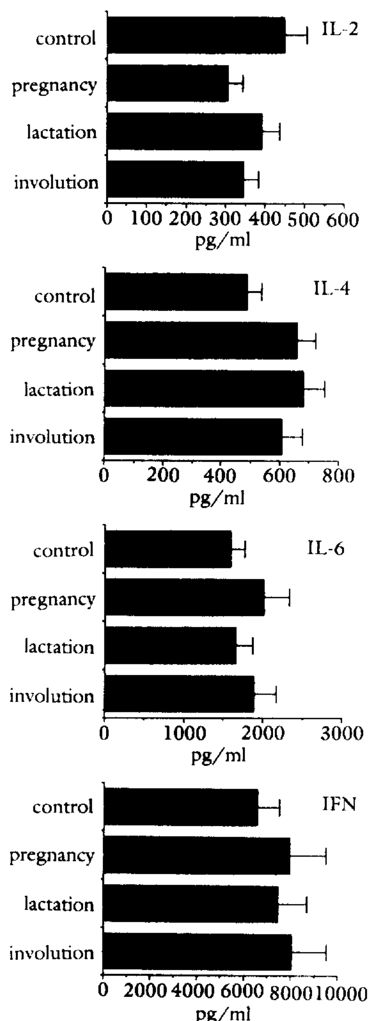

Fig 3. The pattern of lymphokines produced by in vitro activated T cells obtained from the mesenteric lymph nodes. Data are mean±SEM.

various stage of lactation resulted in a reduced capacity to produce the lymphokine IL-2 during the pregnancy and lactation periods($p<0.05$, respectively) and an enhancement in the production of the lymphokines IL-4, IL-6 and IFNγ during the pregnancy and involution periods($p<0.05$, respectively) except the IL-4 during the pregnancy. And especially during the involution period IL-6 was very significantly increased($p<0.01$). These were compared to the pattern of lymphokines produced by activated T cells obtained from mature control mice.

Fig 2 shows that activation-induced production of IL-4, IL-6 and IFNγ were increased in cell cultures of peripheral lymph nodes during the pregnancy and involution periods($p<0.05$, respectively) except the IL-6 during the pregnancy, while the level of IL-2 was decreased during the pregnancy, lactation and, involution periods($p<0.01$, $p<0.05$, $p<0.01$, respectively) when compared control mice.

Fig 3 shows that the production of IL-4 from lymphocytes obtained from the mesenteric lymph nodes was increased during the lactation and pregnancy periods($p<0.05$, respectively), while the level of IL-2 was decreased during the pregnancy($p<0.05$) when compared control mice.

Discussion

Plasma concentrations of several steroid hormones change considerably around the period of gestation and parturition[13,14]. The mammalian immune system has been reported to undergo an impairment of functional immunity on several levels following gestation and parturition[15]. This impairment in immune function has been proposed as a contributor to the increased incidence and severity of diseases.

Lymphocytes obtained from different lymphoid tissues produce different lymphokines follwing activation. An important regulatory factor appears to be the steroid hormones produced in each individual lymphoid tissue[16]. These T cell regulatory hormones include glucocorticoids, dehydroepiandrosterone, dihydrotestosterone, 1,25-dihydroxyvitamin D_3, and others[17]. Therefore, differences in activation-induced production of lymphokines from lymphocytes isolated from different lymphoid tissues during the various stages of lactation under conditions in which the cells would certainly be influenced by changes in the steroid hormone microenvironment *in vivo* might be anticipated.

The studies presented in here have examined both quantitatively and qualitatively aspects of the alternation in lymphokine production by isolated lymphoid cells obtained from female mice, and calculated these with the various stages of lactation. The ability of activated T cells to produce IL-4, IL-6

and IFNγ was increased throughout the lactation cycle when compared with T cells from control donors, while the capacity of T cells to produce IL-2 was decreased. These observations, altering the pattern of lymphokines produced in response to T-cell mitogen stimulation, may be due to the types of steroid hormones capable of having either an enhancing or repressing activity in the control of lymphokine synthesis. Estrogens have been shown to suppress T-cell mediated immunity[18] and glucocorticoids are known inhibitors of IL-2 and IFNγ production by activated murine T cells[9,17]. In contrast, physiologic doses of glucocorticoids are able to enhance IL-4 production[17]. Since IL-2 treatment can enhance the cytotoxic and bactericidal capacity of bovine mammary gland lymphocytes[19] and the intramammary infusion of recombinant bovine IFNγ can prevent the rapid, unrestricted growth of E. coli within the mammary gland[20], it is possible that diminished production of IL-2 and IFNγ may contribute to the periods of heightened susceptibility to intramammary infection. In present studies, the activation-induced production of IFNγ was actually increased above control levels which might be due to an inflammatory change associated with the parturition and the milk retention during the involution period[21,22].

Immediately after parturition, the levels of IL-6 in the whole body fluid of puerperal women is usually considered to increase due to the influence of the IL-6 produced in the placenta and an inflammatory change associated with parturition[22]. Milk retention causes fever and inflammation by the effects of IL-1, tumor necrosis factor α and IL-6 which are contained in the milk[21]. The concentration of IL-6 found in colostrum is also significantly higher than that present in serum or in milk taken 1 month after parturition[22]. IL-6 in whey is derived in part from mononuclear cells[22] and 70-80% of the IL-6 produced by mononuclear cells is derived from monocytes[23]. Therefore, the elevated IL-6 levels in our studies may be due to the continuous challenge by antigens which are present following parturition and weaning. Since the inflammatory activities of IL-6 function to stimulate the immune response to infections[24], further studies need to be undertake in order to better understand the significance and cause of increased levels of IL-6 during the lactation cycle.

Conclusion

The presence of lymphokine activity in the single cell suspensions from peripheral lymph nodes(axillary, brachial, inguinal), mesenteric lymph nodes or spleen of C3H/HeN mice during the various stages of lactation were evaluated. The level of inducible interleukin(IL)-2 activity in splenocytes was lower during the preceding parturition and lactation when compared control mice. During the pregnancy and involution periods, the capacity of stimulated splenocytes to produce IL-4, IL-6 and interferon gamma(IFNγ) were higher than controls. In peripheral lymph nodes, the capacity to produce IL-4, IL-6 and IFNγ were increased during the pregnancy and involution periods, while the capacity to produce the IL-2 was decreased during the pregnancy, lactation and involution periods. In mesenteric lymph nodes, the activation-induced production of IL-4 was increased during the pregnancy and lactation periods, but the level of IL-2 was decreased during the pregnancy. These data suggest that an altered level of inducible lymphokine production during the involution and pregnancy period may correlate with susceptibility to mastitis.

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