Local cytokine levels associated with delayed-type hypersensitivity responses: modulation by gender, ovariectomy, and estrogen replacement

Lisa J Ma, Esther A Guzmán, Ariel DeGuzman, H Konrad Muller, Ameae M Walker and Laurie B Owen

Division of Biomedical Sciences, The University of California, Riverside, California 92521, USA
1Department of Immunology, The University of Texas MD Anderson Cancer Center, Houston, Texas 77030, USA
2Discipline of Pathology, University of Tasmania, Hobart, Tasmania 7000, Australia

(Requests for offprints should be addressed to L J Ma; Email: lma003@student.ucr.edu)

Abstract

It is well established that females mount stronger immune responses than males, but only very little is understood about the underlying mechanisms. We have analyzed local cytokine differences among intact females, those that had been ovariectomized (OVX), those receiving estrogen replacement after OVX, and males, both before and after production of delayed-type hypersensitivity (DTH) responses. We report confirmation of a much larger DTH response in females versus males. However, OVX resulted in an even larger response, while estrogen replacement resulted in a smaller response when compared with intact females. In animals exposed for the first time to an antigen (without a DTH response), OVX increased interleukin-6 (IL-6) and estrogen replacement after OVX suppressed IL-6. Of the cytokines that differed between males and females exposed for the first time to an antigen, only IL-6 was higher in females versus males when exposure to antigen occurred for the second time (when the DTH response occurs). Analysis of cytokines with OVX and estrogen replacement after a second exposure to antigen showed that IL-6 did not significantly change. Levels of IL-4; Regulated upon Activation, Normal T-cell Expressed; and Secreted; and thrombopoietin, however, correlated with the DTH response, suggesting direct or indirect positive regulation by estrogen. These results suggest an important role for both IL-6 and IL-4 in determining the degree of DTH response, with IL-6 (which appears negatively regulated by estrogen) increasing and IL-4 (which appears positively regulated by estrogen) decreasing the response. The results further suggest that IL-6 may play a role in predisposing to a larger DTH response, while IL-4 levels seem more important during an active response.

Introduction

It is generally accepted that females have more robust immune responses after immunization and infection when compared with males (reviewed in Verthelyi 2001), but relatively little is known about how this occurs. In this study, we have used two readily quantifiable inflammatory immune responses, the delayed-type hypersensitivity (DTH) response to Candida albicans and the contact hypersensitivity (CHS) response to fluorescein isothiocyanate (FITC). DTH is elicited by injecting antigen intradermally, while the CHS response, a form of DTH, is induced by epicutaneous application of a sensitizing antigen or hapten (Reeve 2002). Both of these responses are T-cell derived and mediated by cytokines, a form of DTH, is induced by epicutaneous application of a sensitizing antigen or hapten (Reeve 2002). Both of these responses are T-cell derived and mediated by cytokines (Janeway et al. 2001), although different effector cells are responsible for eliciting each. The effector cells involved in the DTH response are local T cells, macrophages, and dendritic cells found at the site into which antigen has been injected. The effector cells involved in the CHS response are the Langerhans cells and the dendritic epidermal T cells found within the epidermis. Langerhans cells have until recently been considered the primary antigen-presenting cell in the skin and have generally been considered to be of importance in the induction of a CHS response (Schwarz 2002). A recent paper by Kaplan et al. (2005), however, suggests an additional modulatory role for Langerhans cells during CHS responses. Dendritic epidermal T cells, which have the gamma delta form of the T-cell receptor (Koning et al. 1987, Steiner et al. 1988), have been shown to be important in controlling inflammation (Girardi et al. 2002), and to play a role in wound healing (Jameson et al. 2002). Cytokines most often associated with down-regulation of inflammatory responses include interleukin-4 (IL-4) and IL-10, while IL-6 and tumor necrosis factor-α (TNF-α) are considered pro-inflammatory (Johnson 1997, Opal & DePalo 2000). In this study, we have examined DTH responses in females that were intact, ovariectomized (OVX), or were OVX with estrogen (E2) replacement, and males to ask which local cytokines are...
implicated as important players in the hormonal regulation of the DTH response.

Materials and Methods

Mice

Pathogen-free male and female C57BL/6 (B6) mice were obtained from the Jackson Laboratories (Bar Harbor, MD, USA). Five-week-old OVX B6 female mice were obtained from the National Cancer Institute (Frederick, MD, USA). Mice were between 10 and 12 weeks of age at onset of all experiments. In total, 188 mice were used for all experiments and their repeats. Animals were housed in cages with controlled temperature and humidity and alternating 12 h light:12 h darkness cycles. The animals were maintained in facilities approved by the Association for the Assessment and Accreditation of Laboratory Animal Care International and in accordance with the current United States Department of Agriculture, Department of Health and Human Services, and National Institutes of Health Regulations. Commercial diet and water were available ad libitum.

Estrogen replacement

Mice were anesthetized using isoflurane (Halocarbon Laboratories, River Edge, NJ, USA) and a small patch of hair was shaved from the scruff. The area was disinfected with alcohol and water were available via free access

DTH response

DTH is a type IV hypersensitivity response. Some mice (46 mice in total) were sensitized with an intradermal injection of fixed C. albicans into both flanks. All mice were anesthetized and some received a second injection 7 days later with C. albicans protein antigen (Alercheck, Inc., Portland, ME, USA) in the footpad. Footpad thickness, a T-cell-mediated response, was measured 24 h (peak footpad thickness) later using a micrometer (Mitutoyo, Tokyo, Japan). Footpads were measured prior to the second exposure and 24-h post-challenge.

GHS response

Mice were shaved on their dorsum and 24 h later some (46 mice total) were sensitized with 0.5% FITC (Sigma) in 1:1 acetone: dibutyl phthalate. Mice were challenged by a second exposure 5 days later on their ears, and the change in ear thickness was measured 24-h post-challenge, previously described as the time of maximal response (Bock & Larsen (1982), using a micrometer (Mitutoyo). Ear thickness was measured prior to the second exposure and 24-h post-challenge. Control animals received FITC on their ears (were challenged) without prior sensitization.

Cytokine array

Mice were killed after measuring footpad thickness and their popliteal lymph nodes isolated. A single cell suspension was obtained and the red blood cells lysed. The resulting cells were used to obtain protein using radio-immunoprecipitation assay (RIPA) cell lysis buffer. Supernatant containing protein were quantified using Bradford assay and 250 µg protein were devoted to cytokine analysis using a RayBio Mouse Cytokine Array (RayBiotech, Inc., Norcross, GA, USA) as directed by their protocol. The cytokines detected by the array were granulocyte colony stimulating factor (CSF), granulocyte macrophage colony stimulating factor (GM-CSF), IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17, interferon-γ (IFN-γ), macrophage chemoattractant protein-1 (MCP-1), MCP-5, Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES), stem cell factor, soluble TNF receptor 1 (sTNFR1), TNF-α, thrombopoietin (TPO), and vascular endothelial growth factor. Densitometric analysis was then performed on the blots using MCID Elite 7.0 software (Imaging Research, Ontario, CA, USA). Background was subtracted and data were normalized against positive controls included on each blot. All data directly compared were derived from the same batch of array membranes.

Statistical analysis

Data were analyzed by ANOVA with post-tests and Bonferroni corrections for multiple comparisons against a single control group on occasions when this was appropriate. The value $P<0.05$ was considered statistically significant. All experiments used four animals per treatment group (except OVX with E2 groups which consisted of three animals per
treatment group). Two individual inflammatory responses were measured on each animal. Therefore, \( n = 8 \) for all groups except OVX with E2 replacement groups where \( n = 6 \). Each experiment was repeated at least twice and each repetition gave similar results. Cytokine array results are presented as the average of two individual experiments, each consisting of pooled samples from four animals (three animals in OVX with E2 group) in which duplicate spots on the array were used for each cytokine measured. All results are presented as means ± s.e.m. Correlations between cytokine levels and DTH responses were determined using the Pearson product-moment coefficient of correlation with the level of significance set at \( P < 0.05 \).

### Results

As expected, both the DTH and CHS responses were 2.5- to 3.0-fold higher in female versus male mice (Fig. 1a and b), thus reproducing a well-documented phenomenon under our experimental conditions. In order to manipulate the endocrine environment, we subjected female animals to OVX or OVX with E2 replacement. There was an increased DTH response 6–8 weeks after OVX. Upon E2 replacement in the OVX animals, the DTH response was decreased below that seen in controls (Fig. 2a). Analysis of the CHS response showed no increase with OVX, but still showed a decreased response upon E2 replacement (Fig. 2b). The inability of OVX to increase the CHS response may be due to maximization of ear thickness, since the maximal change possible appears to be about 0.11 mm (Kawagoe et al. 2002) and ear thickness observed in our study was about 0.1 mm, and thus was already at the top of the range.

Since we obtained opposing effects of OVX and E2 replacement on the DTH response, and the footpad draining lymph nodes are readily identifiable, we focused cytokine analysis on this response. This allowed us to look for cytokines that followed changes in the response. These changes in cytokines could be the result of altered expression or changes in cytokine-producing cell number. First, we analyzed differences between cytokine levels in males and females that had only received a single injection (i.e. under circumstances where immunization, but no inflammation...
had occurred). Of the 22 cytokines measured (see Materials and Methods for complete listing), only IL-6 and MCP-5 were different between the genders (Fig. 3). OVX and OVX with E2 replacement had no effect on MCP-5, whereas OVX produced threefold the level of IL-6 and E2 replacement reduced this, a finding similar in pattern to the overall DTH response.

When a DTH response was induced in males or females, there were significant differences between the genders in the levels of IL-6, IL-9, IL-10, IL-12p40, and IL-12p70, all of which were higher in females (Fig. 4). However, there was no difference in the levels of MCP-5 between the genders (data not shown). Thus, although the levels of MCP-5 were different between males and females before the DTH response, the male mice were capable of equal production once the response was ongoing.

When a DTH response was induced in OVX animals and OVX animals with E2 replacement, we looked for significant changes that followed the pattern or inverse pattern of the DTH response when compared with the intact females. The same pattern (i.e. higher with OVX and reduced in OVX + E2) was observed with sTNFR1, although there was no correlation with the DTH response (R = 0.097). An inverse correlation (i.e. reduced with OVX and higher with OVX + E2) was observed for IL-4 (R = -0.915, P < 0.01), RANTES (R = -0.673, P < 0.05), and TPO (R = -0.992, P < 0.01; Fig. 5). Although IL-12p70 also had an inverse pattern, there was no correlation with the DTH response (R = -0.648). No significant differences were found in the other analytes measured.

Discussion

It is generally accepted that females have more robust immune responses after immunization and infection when compared with males (reviewed in Verthelyi 2001), but relatively little is known about how this occurs. There are many endocrine differences between males and females, which may be responsible for direct or indirect modulation of the immune response including levels of E2, progesterone, testosterone, and their metabolites. Although E2 is higher in females, most articles in the literature have reported an immunosuppressive effect of E2. Thus, for example, E2 has been shown to inhibit the development of experimental autoimmune encephalomyelitis, collagen-induced arthritis, and inflammatory bowel disease (Jansson et al. 1994, Bebo et al. 2001, Ito et al. 2001, Harnish et al. 2004), and to suppress the inflammatory response in castrated mice (Josefsson et al. 1992). Pregnancy, a state of elevated E2 and progesterone, has also been reported to be immunosuppressive (Purttilo et al. 1972, Thong et al. 1973, Kovacs et al. 2002). Findings in the current study are in agreement with these previous reports since we showed an increased DTH response upon OVX and a decreased response upon E2 replacement. Thus, while one can conclude that E2 modulates some immune responses, including those examined in the present study, it is not the higher levels of E2 in females that produces a more robust DTH response.

Others have approached an investigation of the gender difference in immune response by the administration or blockade of various candidate hormones and their metabolites and have uncovered important pro- and anti-inflammatory actions for some of them. E2, for example, is

Figure 3 IL-6 and MCP-5 expression in the challenge only group. Popliteal nodes were obtained from mice that had not been sensitized but only challenged with C. albicans protein. Protein extracted from the nodes was used to perform a cytokine array to measure the levels of cytokines produced. In this graphical representation, data from two separate experiments were combined to allow for ease of comparison. Data are expressed as relative density ± S.E.M. *P < 0.05 compared with male; **P < 0.01 compared with intact female and OVX + E2.

Figure 4 Cytokine expression differs in males and females after sensitization and challenge. Popliteal nodes were obtained from mice sensitized and challenged with C. albicans. Data are expressed as relative density ± S.E.M. *P < 0.05 compared with males.

Figure 5 Cytokine levels significantly altered after sensitization and challenge in OVX and OVX with E2 replacement animals. E2 pellets were inserted into OVX mice 1 week prior to the start of the DTH response experiment. Popliteal nodes were obtained from mice both sensitized and challenged with C. albicans. Data are expressed as relative density ± S.E.M. *P < 0.05 for all cytokines listed.
metabolized to several hydroxyestrogens (reviewed in Mueck et al. 2002), and 4- and 16α-hydroxyestrogens are considered to be pro-inflammatory (Janelle et al. 2006), while 2-hydroxyestrogens are considered to be anti-inflammatory (Janelle et al. 2006). Progesterone is also anti-inflammatory under some circumstances, contributing to tolerance of the embryo during pregnancy (Zhao et al. 2002), and likewise testosterone has been shown to reduce some immune responsiveness (Angele et al. 1997, Wichmann et al. 1997). A previous study in our laboratory demonstrated that E2 replacement in OVX animals resulted in physiological levels of circulating progesterone (Liu et al. 1993). Kinetic analyses conducted by Shimizu et al. (1993) demonstrated that natural estrogens, like estrone and estradiol, inhibit the process of androgen aromatization. In addition, glucocorticoids are anti-inflammatory and OVX has been shown to decrease glucocorticoid secretion and E2 replacement to increase it (Lo et al. 2000, Seale et al. 2004). Remembering that OVX increased and E2 replacement decreased the DTH response, one might suggest therefore that the conversion of E2 to 2-hydroxyestrogens, the stimulatory effect of E2 on progesterone production by the adrenal gland (Janelle et al. 1997), E2 inhibition of aromatase activity in extra-ovarian tissues (Shimizu et al. 1993), or E2 stimulation of glucocorticoid secretion (Lo et al. 2000, Seale et al. 2004) may contribute in some measure to the effects observed. To begin to unravel these complex possibilities, the current study has used the observed different degrees of DTH response in males and females, and manipulation of the latter by OVX and E2 replacement, to uncover important hormonally regulated cytokines, which by their pattern may indicate a significant regulatory role.

First, we duplicated the findings of others concerning gender differences (reviewed in Verthelyi 2001) and showed very clearly that for two different inflammatory responses, females had a far more robust response. In addition, we demonstrated that OVX increased one response and produced no change in the other, the latter most likely due to maximal tissue thickness in the intact animals, which could not be increased further. Consistent with this result and interpretation, E2 replacement in OVX animals decreased both responses to a level below those of intact females. The increased DTH response to OVX and the decreased response upon E2 replacement allowed us to correlate cytokines with the magnitude of these responses as well as with responses in intact females and males.

Analysis of cytokine differences between control males and females after one antigen exposure (i.e. in the absence of a DTH response) suggested a potentially important role for both MCP-5 and IL-6 in determining the magnitude of the DTH response. However, after a second exposure to antigen, MCP-5 levels were no different between males and females, thereby suggesting that MCP-5 levels were not a governing aspect of the degree of response. This conclusion was substantiated by the failure of OVX (which increases the DTH response) or OVX with E2 replacement (which decreases the DTH response) to influence the level of MCP-5. IL-6, on the other hand, showed higher levels in females both before and after the second exposure to antigen (compare Figs 3 and 4), thereby suggesting a more important role for this cytokine. In addition, OVX increased levels of this cytokine, and E2 replacement in OVX animals decreased them, although not lower than those in intact animals. Thus, changes in the level of this cytokine follow expectations for an important positive regulator of the DTH response. Higher levels of IL-6 would be expected to result in a faster onset of the acute phase reaction (Heinrich et al. 2003) and hence a larger DTH response.

Of the cytokines whose levels changed in a similar or inverse pattern to the degree of the DTH response in the OVX and OVX with E2 replacement animals, only IL-12p70 was also significantly different between males and females post-DTH response. IL-12p70 is considered a pro-inflammatory cytokine (Sypek et al. 1993, Romani et al. 1994, Heinzel et al. 1995), but levels were decreased by OVX when the DTH response was increased, and increased upon E2 replacement when the DTH response was decreased. Thus, levels of this cytokine suggest either direct or indirect regulation by E2. However, Pearson’s analysis showed no correlation between the levels of this cytokine and the DTH response. The results therefore do not suggest that IL-12p70 is a cytokine which plays a major local role in governing the degree of the DTH response.

Other cytokines not different between males and females, but which nevertheless show patterns after the DTH response consistent with regulation by E2, include IL-4, sTNFR1, RANTES, and TPO.

IL-4 is an anti-inflammatory cytokine found to be essential for immunosuppression (Souza et al. 2004). Levels of this cytokine were decreased after OVX and increased after E2 replacement, just as one would expect for an anti-inflammatory cytokine, should it be playing an important role in modulating the DTH response; moreover, levels of IL-4 correlated with the DTH response. However, although E2 replacement was able to restore levels of IL-4 to those observed in intact female mice, they did not go higher than intact females and hence restoration of IL-4 cannot completely explain the degree to which the OVX immune responses were suppressed by E2 replacement. This suggests that IL-4 alone is not the sole cytokine responsible for E2 suppression of the DTH response, but the results certainly suggest an important contributory role.

The pattern in sTNFR1 roughly followed the DTH response since amounts were higher after OVX and appeared reduced by E2 replacement, but Pearson’s analysis showed no correlation. Soluble TNFR1 has been shown to induce the cell adhesion molecules ICAM-1 (intercellular adhesion molecule-1), E-selectin, and VCAM-1 (vascular cell adhesion molecule-1) (Amrani et al. 2000, Kitakata et al. 2002). Increased levels of sTNFR1 upon OVX may thus result in increased levels of these cell adhesion molecules, thereby
increasing the migration of cells into the region of inflammation.

The other cytokines showing patterns consistent with positive regulation by E₂, and correlating with the DTH response in the post-DTH group were RANTES and TPO. RANTES has been shown to play a role in DTH responses, but would normally be considered pro-inflammatory (Devergne et al. 1994). The reduction in local levels of RANTES in response to OVX (i.e. when the DTH response was increased) therefore is opposite to what one would predict should it be playing an important role in the response. Similarly, TPO is associated with inflammatory reactions (Burstein et al. 2005) and yet is reduced after OVX. Given the current knowledge, this suggests that RANTES and TPO have a less important role in governing the degree of a DTH response.

The ability of estrogen (either directly or indirectly) to regulate the levels of IL-4, the IL-12p35 and IL-12p40 subunits of IL-12p70, RANTES, and TPO led us to examine the promoter and intronic regions of the genes for these cytokines for consensus response elements (NCBI BLAST). Also examined was the IL-6 gene. The genes for IL-4, both subunits of IL-12p70 and IL-6, each contained likely consensus sequences for estrogen receptor binding. The gene for the p40 subunit of IL-12p70 contained a potential androgen response element, a glucocorticoid response element and a partial progesterone response element. The RANTES gene contained an androgen response element and a glucocorticoid response element. The genes for the p35 subunit of IL-12p70, IL-4, IL-6, and TNFR1 contained potential glucocorticoid response elements. None of the response elements examined were found in the gene for TPO. This suggests that the expression of IL-4, IL-12p35, and IL-12p40 may be directly stimulated by estrogens, while regulation of expression of IL-6, which is higher in females than in males is apparently negatively regulated by E₂. This is in agreement with work in vitro showing direct repression of the IL-6 promoter by E₂ (Pottratz et al. 1994, Ray et al. 1994, Stein & Yang 1995, Galien et al. 1996). Regulation of RANTES, TPO, and sTNFR1 may be more complex.

Study of the endocrinology of estrogen and testosterone has been aided by the development of mice lacking estrogen receptors or having mutations in the androgen receptor. Interpretation of changes in DTH responses in these mice, however, would be complex since estrogen receptor knockout mice have been shown to have altered immune development as well as function (Staples et al. 1999, Thurmond et al. 2000). No known studies on the immunity of androgen knockout mice have been published so far, but one might expect similar complications to interpretation. Future analyses of the role of androgens could benefit from examination of the effect of dihydrotestosterone in orchectomized animals.

In conclusion, our results have demonstrated that the DTH response in particular is a readily quantifiable in vivo assay that can be used to further analyze at least some aspects of hormonal regulation. The data derived from the current study suggest an important role for IL-6 and IL-4 in regulating the degree of the DTH response. Although IL-6 is higher in females than males, it appears to be negatively regulated by E₂. Results obtained for IL-4 on the other hand are consistent with positive regulation of this cytokine by E₂. The results further suggest that IL-6 levels may play a role in predisposing to a larger DTH response, while IL-4 levels seem to be more important during an active DTH response.

Funding

This work was supported by the NIH grant R01CA52457 to L B O and a grant from the Cancer Federation to A M W. L J M was partially supported by a Burden fellowship. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References


Bebo BF Jr, Fyfe-Johnson A, Allard K, Beam AG, Vandenbark AA & Offner BO and a grant from the Cancer Federation to A M W. L J M was partially supported by a Burden fellowship. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References


Bebo BF Jr, Fyfe-Johnson A, Allard K, Beam AG, Vandenbark AA & Offner
Henzel FP, Rkero RM, Ahmed F & Pearlman E 1995 Endogenous IL-12 is required for control of Th2 cytokine responses capable of exacerbating leishmaniasis in normally resistant mice. Journal of Immunology 155 730–739.


Souza VM, Jacysyn JF & Macedo MS 2004 IL-4 and IL-10 are essential for immunosuppression induced by high molecular weight proteins from Ascariis suum. Cytokine 28 92–100.


Received in final form 26 February 2007

Accepted 28 February 2007

Made available online as an Accepted Preprint 6 March 2007

Modulation of DTH by gender, Ovx and E2 · L Ma and others 297

www.endocrinology-journals.org