Raloxifene- and estradiol-mediated effects on uterus, bone and B lymphocytes in mice

M C Erlandsson, C A Jonsson, M K Lindberg¹, C Ohlsson¹ and H Carlsten

Department of Rheumatology and Inflammation Research, University of Göteborg, Guldhedsgatan 10A, S-413 46 Göteborg, Sweden

¹Research Center for Endocrinology and Metabolism, Department of Internal Medicine, University of Göteborg, Göteborg, Sweden

(Requests for offprints should be addressed to M C Erlandsson; Email: malin.erlandsson@rheuma.gu.se)

Abstract

Raloxifene is a selective estrogen receptor modulator approved for the prevention of osteoporosis in postmenopausal women. It is selective by having estrogen-agonistic effects on bone, vessels and blood lipids while it is antagonistic on mammary and uterine tissue. Our aim was to study the agonistic and antagonistic properties of the raloxifene analogue LY117018 (LY) on uterus, bone, B lymphopoiesis and B cell function.

Oophorectomized and sham-operated animals were treated with s.c. injections of equipotent anti-osteoporotic doses of 17 β -estradiol (E2) (0.1 mg/kg) or LY (3 mg/kg) or vehicle as controls. Effects on bone mineral density (BMD) were studied using peripheral quantitative computed tomography, uterine weight was examined,

B lymphopoiesis was examined using flow cytometry and B cell function in bone marrow and spleen was studied by the use of an ELISPOT assay.

E2 and LY had similar effects on BMD and bone marrow B lymphopoiesis, while LY had a clear antagonistic effect on endogenous estrogen in uterine tissue and no stimulating effect on the frequency of Ig-producing B cells in sham-operated animals. Our results are discussed in the context of estrogen receptor biology, relations between the immune system and bone metabolism and also with respect to the estrogen-mediated effects on rheumatic diseases.

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Introduction

Raloxifene is a selective estrogen receptor modulator (SERM) approved to prevent and treat osteoporosis in postmenopausal women (Delmas *et al.* 1997). It is selective by having estrogenic effects on blood lipids and bone formation (Delmas *et al.* 1997), while it is antagonistic on breast tissue (Cummings *et al.* 1999) and uterus (Black *et al.* 1994, Sato *et al.* 1996). Women with an accelerated risk of developing cancer in these tissues can therefore use raloxifene to prevent or treat osteoporosis.

The immune system is influenced by estradiol (E2) in a diverse manner. Removal of estrogen by means of oophorectomy enhances the number of B lymphopoietic cells in bone marrow in mice (Masuzawa *et al.* 1994) and, if E2 is given back, B lymphopoiesis is down-regulated, now with a shift towards more mature B cells (Medina *et al.* 2000). The erythroid and myeloid precursors are not altered after estrogen exposure (Medina *et al.* 1993). The effect of estrogen on B cells is recognized as enhanced Ig production in the rat (Dahlgren & Hanson 1991) and the mouse (Nilsson & Carlsten 1994). In addition, E2 induces dramatic thymic atrophy, including a shift towards more

mature thymocytes (Rijhsinghani *et al.* 1996), and inhibits natural killer cell-mediated cytotoxicity (Hanna & Schneider 1983), and suppresses granulocyte- (Josefsson *et al.* 1992) and T cell-mediated (Carlsten *et al.* 1996) inflammation. The immune-regulating properties of estrogen can influence the outcome of certain autoimmune diseases. Thus, endogenous or added estrogen accelerates the progression of immune complex-mediated murine (Carlsten *et al.* 1990) and human (Ostensen 1999) systemic lupus erythematosus (SLE) but ameliorates T cell- and macrophage-mediated experimental (Holmdahl *et al.* 1989) and human rheumatoid arthritis (RA) (Ostensen 1999).

Bone resorption following estrogen deficiency is primarily caused by increased numbers of bone-resorbing osteoclasts (Pacifi 1996), which formerly were believed to develop exclusively from stem cells of myelopoietic lineage. However, it has recently been shown that osteoclasts can also be formed from early B cells (Manabe *et al.* 2001, Sato *et al.* 2001).

The effects of raloxifene on the immune system are as yet poorly investigated. Recently, we demonstrated that oophorectomized mice treated with the raloxifene

analogue LY117018 (LY) underwent partial thymic atrophy, but failed to shift thymocytes towards more mature T cells. We also showed that LY lacks the suppressive effects of estrogen on granulocyte- and T cell-mediated inflammation (Erlandsson *et al.* 2000). The aim of the present study was to examine how LY affects B lymphopoiesis in the bone marrow and the function of B cells in bone marrow and spleen. We treated both oophorectomized and sham-operated animals with LY, E2 or vehicle control in order to identify areas where raloxifene acts in an antagonistic and/or an agonistic manner compared with endogenous estrogen.

Materials and Methods

Mice

C57BL/6 mice were bought from Bomholtgård Ltd (Ry, Denmark). They were housed five to ten animals in each cage under standard conditions of temperature and light and fed standard laboratory chow *ad libitum*.

Castration

All mice were castrated at the age of 5–6 weeks. Ovaries were removed after a flank incision and the incisions were closed with metallic clips. The oophorectomy was carried out under Hypnorm/Dormicum anesthesia. Sham-operation was carried out in a manner similar to oophorectomy, but excluding the removal of the ovaries. Mice were left to rest for 1 week after operation before the start of the experiments. The experiments were approved by the local ethics committee for animal research.

Hormones and treatment

The hormones used were 17 β -estradiol benzoate (E2; Sigma, St Louis, MO, USA) and the raloxifene analogue LY117018 free base (LY), kindly donated by Eli Lilly Corporation, Indianapolis, IN, USA. Raloxifene is a non-steroid hormone based on benzothiophene. LY differs from raloxifene at only one site in the molecule: instead of a pyrrolidine ring it has a piperidine ring on the basic side chain. The mice were treated, as previously described (Erlandsson *et al.* 2000), with s.c. injections of 3 mg LY/kg, or 0.1 mg E2/kg dissolved in 100 μ l olive oil (Apoteksbolaget, Göteborg, Sweden) for 5 days/week for 2.5 weeks. Controls were given vehicle (100 μ l olive oil/mouse) for 5 days/week. The doses were chosen as equipotent to those for treating osteoporosis (Sato *et al.* 1995).

Cellular parameters

Tissue collection and single cell preparation The mice were killed by cervical dislocation. Uteri and spleens

were autopsied and weighed. Spleen single cell suspensions were obtained after tissue was mashed and passed through a nylon wool sieve. The cells were centrifuged at 515 g for 5 min and pelleted spleen cells were resuspended in Tris-buffered 0.83% NH₄Cl solution (pH 7.29) to lyse erythrocytes. Bone marrow cells were harvested from the cavity of the right femur using a syringe with 2 ml phosphate-buffered serum (PBS). Cells were kept in complete Iscove's medium (Invitrogen, Carlsbad, CA, USA)/PBS (50/50) until use. After being washed in PBS, the total number of leukocytes was calculated, using an automated cell counter (Sysmex, Kobe, Japan), the cells were subjected to Fluorescence Activated Cell Sorting (FACS) analysis and an Enzyme Linked ImmunoSPOT (ELISPOT) assay to determine the number of Ig-producing cells.

Flow cytometry for analysis of cell phenotypes Isolated splenocytes were stained with fluorescein isothiocyanate (FITC)-labeled antibodies to CD45R/B220 (clone RA3-6B2) (Beckton-Dickinson, Franklin Lakes, NJ, USA) and phycoerythrin (PE)-conjugated antibodies to CD3 ϵ (clone 145-2C11) (Beckton-Dickinson). Bone marrow cells were labeled with α CD45R/B220-FITC and PE-conjugated antibodies to the immunoglobulin heavy chain μ (clone 1021-09) (Southern Biotechnology Inc., Birmingham, AL, USA). All cells were analysed in a FACSCalibur (Beckton-Dickinson). Stained cells were expressed as percentage of gated mononuclear cells.

ELISPOT The ELISPOT technique (Czerkinsky *et al.* 1984) was used for evaluation of IgG-, IgM- and IgA-secreting bone marrow and spleen cells. Briefly, 96-well nitro-cellulose plates (Millipore, Bedford, MA, USA) were coated with 100 μ l PBS containing 5 μ g/ml affinity purified F(ab')₂ fragments of goat anti-mouse IgG, IgM and IgA (Southern Biotechnology Inc.). After overnight incubation at 4 °C and blocking with 5% fetal calf serum, 50 μ l Iscove's culture medium containing 10⁶ or 10⁵ freshly isolated spleen or bone marrow cells was added to each well. The plates were incubated for 3.5 h at 37 °C in 5% CO₂ and 95% humidity. After being rinsed, each well was incubated stepwise with 100 μ l alkaline phosphatase-conjugated goat anti-mouse IgG, IgM or IgA diluted in 1:750 PBS and finally with the substrate 5-bromo-4-chloro-3-indolyl phosphate (Sigma). After 1 h, the reaction was stopped by washing in tap water and each well was examined for the appearance of dark blue spots. The samples were run in triplicate and the numbers of Ig-secreting cells were expressed as the frequency of spot-forming cells (SFC)/10³ B220⁺ cells.

Histological parameters

Peripheral quantitative computed tomography (pQCT) Tomographic measurements were made using the Stratec pQCT XCT Research M specifically modified

for use on small bone specimens (software version 5.4B; resolution 70 µm; Norland, Fort Atkinson, WI, USA). *Ex vivo* metaphyseal pQCT scans of the distal metaphyseal part of the left femur were used to measure bone mineral density (BMD) as previously described (Vidal *et al.* 2000).

Serological parameters

Serum Blood was collected at the termination of the experiments. The sera were individually stored at -20°C until use.

E2 radioimmunoassay (RIA) Serum E2 levels were measured using an E2 RIA (DSL-39100) scaled down 1:4 (Diagnostic Systems Laboratories Inc., Webster, TX, USA). The sensitivity of the assay is less than 1 pg/ml.

Insulin-like growth factor-I (IGF-I) RIA Serum IGF-I levels were measured by double-antibody IGF-binding protein-blocked RIA, according to Blum & Brier (1994).

Statistical analysis

Unpaired *t*-test was used for statistical comparisons between means of different treatment groups. Results are presented as means $\pm$ S.E.M. A *P* value <0.05 was considered statistically significant.

Results

Effects on uterus and bone

Oophorectomized (OVX) and sham-operated mice were treated with E2 or LY in doses equipotent with those used for the prevention of osteoporosis. In both OVX and sham-operated groups, exposure to E2 resulted in similar and highly elevated uterus weights, whereas OVX control animals displayed low uterus weights with very small inter-individual variations (Table 1). In both OVX and sham-operated groups, treatment with LY induced intermediate uterus weights, again with small inter-individual variations. Thus, exposure to LY reduces uterus weight in sham-operated animals but increases it in OVX animals, clearly demonstrating both antagonistic and agonistic effects of LY in the uterus. Serum levels of E2 were measured after the end of the experiment and the outcome was as expected, low levels in non-treated and LY-exposed sham-operated animals, significantly lower in OVX animals and significantly higher in both E2 treatment groups (Table 1).

The distal metaphyseal part of the femur was subjected to pQCT analysis of BMD. As expected, a significant reduction in BMD was found upon OVX (Table 2). Exposure to both E2 and LY induced a significant increase

Table 1 Uterus weight and serum E2 levels of mice after 2.5 weeks of hormone treatment. Values are means $\pm$ S.E.M.

Operation	Treatment	<i>n</i>	Uterus weight (mg)	Serum E2 level (pg/ml)
Sham	Controls (C)	6	92 $\pm$ 19	12 $\pm$ 3
	C vs E2		ns	**
	0.1 mg E2/kg	6	164 $\pm$ 28	58 $\pm$ 12
	E2 vs LY		**	**
	3 mg LY/kg	6	53 $\pm$ 1	13 $\pm$ 1
OVX	C vs LY		ns	ns
	Controls	10	18 $\pm$ 1 ^{†††}	6 $\pm$ 1 [†]
	C vs E2		***	***
	0.1 mg E2/kg	10	155 $\pm$ 19	62 $\pm$ 13
	E2 vs LY		***	***
	3 mg LY/kg	10	35 $\pm$ 2 ^{†††}	8 $\pm$ 2
	C vs LY		***	ns

P* < 0.01, *P* < 0.001.

[†]*P* < 0.05, ^{†††}*P* < 0.001 (OVX vs sham).

ns, not significant.

in BMD in sham-operated and OVX animals (Table 2 and Fig. 1). The effect of E2 treatment was, however, slightly but significantly larger than the effect of LY treatment in both sham-operated and OVX animals.

Effects on bone marrow B lymphopoiesis

Bone marrow cells from OVX or sham-operated mice treated for 2.5 weeks with E2 or LY were labeled with monoclonal antibodies to B220⁺ and the IgM μ -chain and subjected to FACS analysis. In control mice, OVX resulted in an increased frequency of B lymphopoietic (B220⁺) cells. In both the OVX and the sham-operated group, treatment with both E2 and LY resulted in decreased frequency of B220⁺ cells (Fig. 2).

The B220⁺ cells were divided into four groups in order of appearance in the B cell maturation: B220^{low} μ^{-} (1), B220^{low} μ^{+} (2), B220^{high} μ^{+} (3) and B220^{high} μ^{-} (4).

Table 2 BMD of the distal metaphyseal part of the femur of mice after 2.5 weeks of hormone treatment. Values are means $\pm$ S.E.M.

Treatment	Sham (mg/cm ³)	<i>n</i>	<i>P</i> (sham vs OVX)	OVX (mg/cm ³)	<i>n</i>
Controls (C)	460 $\pm$ 15	6	***	371 $\pm$ 6	10
C vs E2	***			***	
0.1 mg E2/kg	598 $\pm$ 17	6	ns	574 $\pm$ 15	10
E2 vs LY	**			*	
3 mg LY/kg	511 $\pm$ 16	6	*	523 $\pm$ 10	10
C vs LY	*			***	

P* < 0.05, *P* < 0.01, ****P* < 0.001.

ns, not significant.

Figure 1 Representative pQCT scans of the distal metaphysis of the femur of OVX or sham-operated mice treated 5 days/week for 2.5 weeks with 0.1 mg/kg E2, 3 mg/kg LY or olive oil as controls. Pictures are representatives from each group consisting of six to ten mice/group. Means $\pm$ S.E.M. are shown in Table 2.

Table 3 shows that OVX resulted in decreased frequency of mature B220^{high} B cells whereas the treatment of sham-operated and OVX mice with E2 or LY induced a significant shift of B lymphopoietic cells from immature B220^{low} cells to more mature B220^{high} cells. Representative FACS plots are shown in Fig. 3.

Bone marrow cells were subjected to an Ig isotype-specific ELISPOT assay for evaluation of Ig-producing cells. Exposure to E2 increased the frequency of Ig-producing cells (measured as Ig SFC/10³ B220⁺ cells) in both sham-operated animals and the OVX group (Fig. 4). In contrast, a similar increase in frequency of Ig-producing cells was seen in LY-exposed OVX mice but not in sham-operated animals.

Effects on spleen cells

Spleen cellularity was significantly lower after E2 or LY treatment in both the OVX and the sham-operated animals (Table 4). Splenocytes from all treatment groups were labeled with monoclonal antibodies to B220 and CD3 and subjected to FACS analysis. After OVX, both hormones induced a lower frequency of B220⁺ cells in spleen, a phenomenon not seen in sham-operated mice (Table 4). When the number of B220⁺ spleen cells was calculated, both treatments resulted in a lower number on both backgrounds (sham-operated and OVX) (Table 4). A similar result was found for the number of CD3⁺ spleen cells (data not shown).

Spleen cells were subjected to an Ig isotype-specific ELISPOT assay for evaluation of Ig-producing cells. In OVX animals, E2 treatment led to a higher frequency of Ig-producing B220⁺ cells, while LY treatment did not alter the frequency. Sham-operated animals had significantly higher frequencies of Ig upon treatment with E2, but not after treatment with LY (Fig. 5).

Effects on serum IGF-I levels

In OVX mice, treatment with E2 resulted in slightly decreased serum IGF-I levels not seen in LY-exposed mice. In contrast, sham-operated animals treated with LY displayed significantly higher IGF-I levels, whereas exposure to E2 did not alter IGF-I levels, possibly indicating the antagonistic properties of raloxifene (Table 5).

Discussion

The aim of the present report was to study the agonistic and antagonistic properties of the raloxifene analogue LY117018 to endogenous estrogen in uterus, bone, on B lymphopoiesis in bone marrow and on B lymphocytes in spleen.

It was previously shown that raloxifene exerts a minor agonistic effect on the uterus in castrated rats (Black *et al.* 1994). We have now shown not only a weak agonistic

Table 3 B lymphopoietic phenotypes (%) in mice treated with E2 or LY for 2.5 weeks. Values are means $\pm$ S.E.M.

	Treatment	n	B220 ^{low} μ^- ⁽¹⁾	B220 ^{low} μ^+ ⁽¹⁾	B220 ^{high} μ^+ ⁽¹⁾	B220 ^{high} μ^- ⁽¹⁾
Operation						
	Sham					
	Controls (C)	6	43.8 $\pm$ 2.8	36.3 $\pm$ 1.7	16.2 $\pm$ 2.5	3.7 $\pm$ 0.9
	C vs E2		*	***	***	ns
OVX						
	0.1 mg E2/kg	6	32.6 $\pm$ 2.5	19.3 $\pm$ 1.5	41.1 $\pm$ 3.9	7.0 $\pm$ 1.2
	E2 vs LY		ns	ns	ns	ns
	3 mg LY/kg	6	26.9 $\pm$ 2.2	19.2 $\pm$ 1.2	47.9 $\pm$ 2.8	6.0 $\pm$ 0.2
	C vs LY		***	***	***	*
OVX						
	Controls	10	58.5 $\pm$ 2.0 ^{‡‡}	30.2 $\pm$ 0.8 ^{‡‡}	10.3 $\pm$ 1.5 [‡]	1.0 $\pm$ 0.1 ^{‡‡}
	C vs E2		***	***	***	***
	0.1 mg E2/kg	10	39.0 $\pm$ 2.9	14.4 $\pm$ 1.2 [‡]	37.0 $\pm$ 3.3	9.6 $\pm$ 1.0
OVX						
	E2 vs LY		**	ns	**	ns
	3 mg LY/kg	10	49.5 $\pm$ 1.8 ^{‡‡}	17.6 $\pm$ 1.9	24.5 $\pm$ 1.7 ^{‡‡‡}	8.4 $\pm$ 0.6 ^{‡‡}
	C vs LY		**	***	***	***

⁽¹⁾% of B220⁺ bone marrow cells.

P*<0.05, *P*<0.01, ****P*<0.001.

[‡]*P*<0.05, ^{‡‡}*P*<0.01, ^{‡‡‡}*P*<0.001 (OVX vs sham).

ns, not significant.

effect of LY on OVX mice but also a clear antagonistic effect on sham-operated animals (Table 1). In a recent study, using low doses of E2 and raloxifene in normal mice, the uterus weight was decreased, but not enough to be significant (Onoe *et al.* 2000). A study on ovulating women revealed only minor antagonistic effects on their endometrial biopsy specimens after raloxifene treatment (Baker *et al.* 1998). We therefore believe that raloxifene acts as a competing antagonist to endogenous estrogen at the estrogen receptor (ER) level in the uterus. This is supported by studies carried out *in vitro* where endometrial cancer cells were treated with a combination of E2 and raloxifene (Kleinman *et al.* 1996).

Castration of both males and females induces osteoporosis. Physiological doses of estrogen restore the BMD whereas osteopetrosis is induced when pharmacologically high doses are given (Samuels *et al.* 1999). Raloxifene has the same properties of bone formation as estrogen, but requires a dose of about 30-fold (Sato *et al.* 1995). In the present study, exposure to both E2 and LY induced a significant increase in BMD in sham-operated and OVX animals (Table 2).

Castration of both male and female mice induces an increased number of nucleated cells in bone marrow. The selectively expanding cell population consists of early B lymphopoietic B220⁺ cells (Masuzawa *et al.* 1994). If estrogen is replaced, the frequency of B220⁺ cells is reduced in a dose-dependent manner (Onoe *et al.* 2000) and there is a shift towards higher frequencies of more mature B cells (Medina *et al.* 2000). We have now shown that exposure to LY induces the same pattern of shift, independent of hormonal background (sham-operated or OVX) (Fig. 3 and Table 3). Recently Onoe *et al.* (2000) presented a similar shift of B cell frequencies in OVX animals treated with raloxifene but, in contrast to our

work, when treating sham-operated mice they did not find any difference between controls and raloxifene-treated animals, possibly due to lower treatment doses.

The effects of LY treatment shown above are similar to the known E2-induced effects on both bone and B lymphopoiesis. Bone metabolism is a delicate balance

Figure 2 The percentage of B220⁺ cells in bone marrow of OVX or sham-operated mice treated 5 days/week for 2.5 weeks with 0.1 mg/kg E2 (shaded bars), 3 mg/kg LY (open bars) or olive oil as controls (solid bars). Bars represent means and S.E.M. **P*<0.05, ***P*<0.01, ****P*<0.001 (treatment versus controls); ^{‡‡}*P*<0.01, ^{‡‡‡}*P*<0.001 (OVX versus sham-operated).

Figure 3 Effects on B220⁺ sub-populations in bone marrow of OVX or sham-operated mice treated 5 days/week for 2.5 weeks with 0.1 mg/kg E2, 3 mg/kg LY or olive oil as controls. The sub-populations are numbered in order of appearance in B cell maturation: (1) B220^{low}μ⁻, (2) B220^{low}μ⁺, (3) B220^{high}μ⁺ and (4) B220^{high}μ⁻. Pictures are representative plots of six to ten mice in each group. Means ± S.E.M. are shown in Table 3.

of bone-forming osteoblasts and resorbing osteoclasts. The postmenopausal osteoporosis induced by estrogen deficiency is primarily caused by an increased osteoclast formation (Pacifci 1996). Osteoclasts were formerly believed to differentiate only from stem cells of the myeloid lineage but recently osteoclasts have been shown also to differentiate from B lymphocyte precursors in the bone marrow (Manabe *et al.* 2001). It is suggested that this new osteoclast differentiation pathway has a role in osteoporosis caused by estrogen deficiency (Sato *et al.* 2001). It has also been shown that T lymphocytes producing tumor-necrosis factor- α have a role in the up-regulation of osteoclast production following OVX (Roggia *et al.* 2001). Taken together, the bone metabolism and the differentiation of lymphoid cells are more closely connected than was formerly believed.

We further investigated the effects of LY on mature B cell function by the use of an ELISPOT assay evaluating Ig-producing B cells in bone marrow and spleen. Estrogen treatment is known to increase the frequency of B cells actively producing Ig (Nilsson & Carlsten 1994) and, as expected, E2 induced higher frequencies of B lymphocytes

producing Ig in both OVX and intact animals. Treatment with LY though failed to increase the frequency of spot-forming B-cells in sham-operated mice, while in OVX animals the outcome of LY treatment was similar to E2 treatment. The same pattern was found in both bone marrow (Fig. 4) and spleen (Fig. 5). Thus, raloxifene acts as an agonist on the OVX background, but not on the naïve background.

The spleen cellularity was decreased after both E2 and LY exposure as previously shown (Erlandsson *et al.* 2000, 2001). We have now shown that the percentage of B lymphocytes in spleen decreased after exposure to both E2 and LY in OVX animals but not in sham-operated mice. The mechanism whereby estrogen regulates spleen cellularity and lymphocyte phenotypes is not known. One hypothesis is that these effects are mediated via action on the growth hormone (GH)/IGF-I axis. It has been clearly shown that Snell Dwarf mice, with impaired production of GH, have smaller spleens and lower frequency of B cells in the spleen (Montecino-Rodriguez *et al.* 1997).

IGF-I has a role in up-regulating both B and T lymphopoiesis (Foster *et al.* 1998). IGF-I and E2 have also

Figure 4 Effects on frequency of Ig-producing cells in the bone marrow of OVX or sham-operated mice treated 5 days/week for 2.5 weeks with 0.1 mg/kg E2, 3 mg/kg LY or olive oil as controls (C). Data are shown as Ig SFC/1000 B220⁺ cells in the following order (beginning from the bottom): solid, IgM; shaded, IgG; open, IgA. Bars represent means and S.E.M. for the summation of IgM, IgG and IgA. **P*<0.05, ****P*<0.001, ns, not significant.

Figure 5 Effects on frequency of Ig-producing cells in the spleen of OVX or sham-operated mice treated 5 days/week for 2.5 weeks with 0.1 mg/kg E2, 3 mg/kg LY or olive oil as controls. Data are shown as Ig SFC/1000 B220⁺ cells in the following order (beginning from the bottom): solid, IgM; shaded, IgG; open, IgA. Bars represent means and S.E.M. for the summation of IgM, IgG and IgA. **P*<0.05, ***P*<0.01, ns, not significant.

been shown to regulate the expression of each other's receptors (Kahlert *et al.* 2000, Stoica *et al.* 2000). In addition, E2 regulates the pituitary expression of GH, which accomplishes many of its effects through IGF-I, mainly expressed in the liver. Treatment of OVX mice with E2 tended to decrease serum levels of IGF-I (Table 5) as previously shown (Erlandsson *et al.* 2001). LY did not affect the serum IGF-I levels of OVX mice. In sham-

operated animals, E2 had no effect while LY increased the IGF-I level, possibly indicating an antagonistic action of raloxifene on systemic IGF-I levels.

The above data suggest different effects of raloxifene exposure on different tissues or cell types. A possible mechanism for these effects is the different tissue distribution of the two known ERs, ER- α and ER- β . For example, ER- α is the dominant form expressed in the

Table 4 Number of spleen cells and frequency of cell phenotypes in spleens of mice treated with E2 or LY for 2.5 weeks. Data are presented as percentage of gated mononuclear cells. Values are means $\pm$ S.E.M.

	Treatment	<i>n</i>	Millions of spleen cells	% B220 ⁺ cells	Millions of B220 ⁺ splenocytes
Operation Sham	Controls (C)	6	169 $\pm$ 22	58.4 $\pm$ 3.3	95.3 $\pm$ 7.4
	C vs E2		*	ns	**
	0.1 mg E2/kg	6	112 $\pm$ 6	54.7 $\pm$ 0.9	61.8 $\pm$ 4.3
	E2 vs LY		ns	ns	ns
	3 mg LY/kg	6	98 $\pm$ 9	55.9 $\pm$ 4.0	55.4 $\pm$ 4.3
	C vs LY		*	ns	**
OVX	Controls (C)	10	224 $\pm$ 18	53.5 $\pm$ 2.0	117.6 $\pm$ 7.2
	C vs E2		*	***	***
	0.1 mg E2/kg	10	153 $\pm$ 24	37.5 $\pm$ 2.0 ^{††}	55.5 $\pm$ 6.6
	E2 vs LY		ns	ns	ns
	3 mg LY/kg	10	130 $\pm$ 10 [‡]	36.8 $\pm$ 1.5 ^{†††}	47.6 $\pm$ 3.5
	C vs LY		***	***	***

P*<0.05, *P*<0.01, ****P*<0.001.

[‡]*P*<0.05, ^{†††}*P*<0.001 (OVX vs sham).

ns, not significant.

Table 5 Serum levels of IGF-I in mice treated with E2 or LY for 2·5 weeks. Values are means ± S.E.M.

Treatment	Sham (ng/ml)	n	P (sham vs OVX)	OVX (ng/ml)	n
Controls (C)	291 ± 16	6	ns	305 ± 16	10
C vs E2	ns			ns	
0·1 mg E2/kg	297 ± 16	6	ns	271 ± 15	10
E2 vs LY	ns			ns	
3 mg LY/kg	350 ± 20	6	*	298 ± 12	10
C vs LY	*			ns	

*P<0·05.
ns, not significant.

uterus while, in the ovaries, both ER-α and ER-β are highly expressed but with differential cellular distribution (Kuiper *et al.* 1998). Both ERs have also been found to varying extent in bone marrow, spleen and thymus (Couse *et al.* 1997, Onoe *et al.* 1997, Vidal *et al.* 1999).

A three-dimensional (3D) crystal structure of ER-α with raloxifene bound displays how the conformation is changed compared with the 3D structure with E2 (Brzozowski *et al.* 1997). When raloxifene is bound to ER-β a similar effect is found (Pike *et al.* 1999). The change in conformation results in limited ability to bind tissue-specific co-activators needed for full agonistic effect (Brzozowski *et al.* 1997, Lonard & Smith 2002).

Certain autoimmune diseases are affected by endogenous or added estrogen. For instance, estrogen ameliorates T cell and macrophage-mediated RA, but aggravates B cell and immune complex-driven SLE. Patients with both SLE and RA are at risk of developing osteoporosis as a result of chronic inflammation and long-term treatment with corticosteroids. The optimal SERM for patients with SLE should have estrogenic effects on bone formation and inflammation but not on B cells. Future development of ER-α- and ER-β-specific ligands could possibly help to create drugs with properties optimized for use in these patients.

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