Archivum Immunologiae et Therapiae Experimentalis, 2003, **51**, 185–193
PL ISSN 0004-069X

17β-Estradiol Treatment Profoundly Down-Regulates Gene Expression in Spinal Cord Tissue in Mice Protected from Experimental Autoimmune Encephalomyelitis

AGATA MATEJUK[1, 2, 3], JAMI DWYER[2], CORWYN HOPKE[1, 2], ARTHUR A. VANDENBARK[1, 2, 4] and HALINA OFFNER[1, 2*]

[1] Department of Neurology, Oregon Health and Science University, Portland, OR 97201, USA, [2] Neuroimmunology Research, Veterans Affairs Medical Center, Portland, OR 97239, USA, [3] L. Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland, [4] Department of Molecular Microbiology and Immunology, Oregon Health and Science University, Portland, OR 97201, USA

Abstract. It is now well documented that experimental autoimmune encephalomyeltitis (EAE) can be effectively prevented by estrogen therapy. Previously, we identified a limited set of genes that were altered in spleens of mice protected from EAE by 17β-estradiol (E2) treatment. As a continuation of these studies, we present here transcriptional changes in genes expressed in spinal cord tissue. The Affymetrix microarray system was used to screen more than 12,000 genes from E2-treated double transgenic (BV8S2 and AV4) female mice protected from EAE vs. control mice with severe EAE. We found that estrogen therapy had a profound inhibitory effect on the expressions of many immune-related genes in spinal cords. Estrogen significantly affected the transcription of 315 genes, 302 of which were down-regulated and only 13 that were up-regulated by ≥2.4 fold. A number of genes encoding the histocompatibility complex, cytokines/receptors, chemokines, adhesion molecules, and signal transduction proteins were strongly down-regulated (>20 fold) in estrogen-treated mice to levels similar to those of the spinal cord tissue from unmanipulated mice. The identification of genes with altered expression patterns in the spinal cords of estrogen-treated mice provides unique insight into the process that ultimately results in protection against EAE.

Key words: 17β estradiol; EAE; microarray; spinal cord.

Introduction

Experimental autoimmune encephalomyelitis (EAE) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) in rodents that is a useful model for the human disease multiple sclerosis (MS). EAE involves a complex disease process that is initiated by antigen-specific T cells, which are generated in the systemic organs and migrate to the CNS. In the diseased CNS there is a strong up-regulation of inflammation-related molecules and activation of recruited macrophages and resident microglial cells (the major phagocytic and antigen-presenting cells) that ultimately results in myelin damage.

* Correspondence to: Halina Offner, D.Sc., Portland VA Medical Center, Neuroimmunology Research R&D-31, 3710 SW US Veterans Hospital Rd., Portland, OR 97239, USA, tel.: +1 503 721 7893, fax: +1 503 721 7975, e-mail: offnerva@ohsu.edu

Previously we demonstrated that treatment with 17β-estradiol (E2) can ameliorate clinical and histological signs of disease. E2 treatment inhibited the capacity for developing myelin-reactive T cells and down-regulated cytokine/chemokine mRNA expression in the CNS[3, 19]. We further demonstrated that one of the key inflammatory cytokines affected by E2 was tumor necrosis factor α (TNF-α)[15]. Although the beneficial anti-inflammatory effects of E2 therapy have been well documented, the molecular basis for protection against EAE is still not well understood. Recently we evaluated transcriptional changes in splenocytes from E2-treated and control T cell receptor (TCR) transgenic female mice with EAE, using DNA microarray technology. From this study, we identified a limited number of E2-sensitive immune-related genes that were either down-regulated, including TNF-α and the chemokine RANTES, or up-regulated, including cytotoxic T lymphocyte antigen 4 (CTLA-4), transforming growth factor β3 (TGF-β3), interleukin 18 (IL-18), two interferon γ (IFN-γ)-induced genes, the chemokines, monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 1β (MIP-1β), vascular cell adhesion molecule (VCAM), and disintegrin metalloprotease, among others[20].

In the current study, we now present new data obtained from the target organ of inflammation – the spinal cord (SC) – at the peak of disease in E2-protected and control mice with severe EAE. Overall, a similar number of E2-sensitive genes was found in CNS as compared with the spleen. However, unlike splenocytes, the great majority (96.5%) of affected genes (≥2.4 fold change) in the CNS were down-regulated, with only 3.5% being up-regulated after E2 therapy. Moreover, many[17] of the down-regulated genes displayed striking (>20 fold) changes. Overall, the profile of transcriptional changes involved genes related to antigen presentation and processing, signal transduction, transcription regulation, cytokines/receptors, chemokines, adhesion molecules, apoptosis/death, and acute phase/complement activation molecules. The genes in the CNS most affected by E2 treatment approximated the same set of genes up-regulated during EAE, resulting in minimal changes relative to their expressions in spinal cord tissue from unmanipulated mice. This result is consistent with the idea that E2 dampens the inflammatory process systemically such that infiltrating mononuclear cells cannot penetrate the CNS, rather than up-regulating protective CNS gene expression.

Materials and Methods

Mice. Double-transgenic (Tg) female mice bearing the rearranged BV8S2 and AV4 genes on the B10.PL background were kindly provided by Dr. Janeway and were bred in house. The colony was housed and cared for in the Animal Resource Facility at the Portland VAMC according to institutional guidelines. Mice were used at 8–12 weeks of age.

Induction of active EAE. BV8S2/AV4 double-Tg female mice were immunized with 400 μg of MBP-Ac1-11 (Ac-ASQKRPSQRSK) in complete Freund's adjuvant (CFA) containing 200 μg of *Mycobacterium tuberculosis* by subcutaneous (s.c.) injection over four sites on the flank on day 0. Mice were assessed daily for clinical signs of EAE according to the following scale: 0 – no signs; 1 – limp tail; 2 – moderate hind limb weakness (waddling gait); 3 – moderately severe hind limb weakness; 4 – severe hind limb weakness; 5 – paraplegia; 6 – quadriplegia, moribund condition. At the peak/acute phase of EAE (maximum severity of clinical signs lasting for three subsequent days, which consistently occurred between 14–16 days after immunization with encephalitogenic peptide), representative mice from EAE- and estrogen-treated groups were euthanized and the spinal cords were removed. The frozen spinal cords were subsequently subjected to total RNA extraction using the STAT-60 reagent (Tel-Test, Inc., Friendswood, TX, USA).

Estrogen treatment. For estrogen hormone therapy 3 mm pellets containing 2.5 mg of 17β-estradiol (1500–2000 pg/ml serum, 20–50% of pregnancy levels, Innovative Research of America, Sarasota, FL, USA) were implanted s.c. on the back 7 days before induction of EAE. EAE mice were sham operated and were implanted with a control pellet (which did not affect the course of EAE, unpublished data). Unmanipulated mice served as controls. The estrogen pellets provide continuous controlled release of a constant level of hormone over a period of 60 days. Serum concentrations of estrogen monitored before and during the course of EAE in representative control and implanted mice consistently fell within the expected ranges as measured by the radioimmunoassay method.

GeneChip Array Assay. The microarray assays were performed in the Affymetrix Microarray Core of the OHSU Gene Microarray Shared Resource. The detailed methodology of cDNA microarray and data analysis was performed as previously described[20]. Each target was hybridized to an MG_U47A array with 12,000 genes using protocols described in the Affymetrix Expression Analysis Technical Manual. A listing of genes

that are on the chip used in this study can be found at http://snow.prohosting.com/affy.

Data presentation. Data presented in Tables 1 and 2 show mean fold change (decrease or increase, respectively) in gene expression with standard deviation (SD) from three independent experiments in estrogen-treated mice as compared with EAE mice with severe clinical signs of disease. The minimum change in mean fold chosen for presentation was 2.4 (the same as used for spleen) with a SD of no more than 30%. The transcriptional changes in genes of interest in estrogen treated mice over unmanipulated mice are also presented in Tables 1 and 2. The complete list of effected genes (fold change 1.5 and more, SD≤30%) is available online.

Table 1. Down-regulation of cytokines, chemokines, cytokine receptors, chemokine receptors, adhesion molecules, activation markers, apoptosis, signal transduction, and T cell receptor molecules, and other genes of interest

Name	Gene description	**Estrogen over EAE**		Estrogen over naive	
		fold change	**SD**	fold change	SD
Antigen presentation/processing					
H2-Aa	Histocompatibility 2, class II antigen A,	**–203.6**	**54.3**	7.2*	6.2
LMP2, PSMB9	Proteasome β type 9 (large multifunctional protease 2)	**–70.5**	**7.4**	1.6*	4.4
GILT, IP-30, IFI30	Interferon γ inducible protein 30	**–47.3**	**10.2**	1.6*	2.3
H2-DMβ1, Ii	Histocompatibility 2, class II, locus Mβ1	**–35.0**	**6.4**	–0.5*	2.5
H2-Eβ1	Histocompatibility 2, class II antigen Eβ	**–27.7**	**2.1**	3.2*	0.8
H2-T23	Histocompatibility 2, T region locus 23	**–10.4**	**1.8**	2.7	1.0
D2d	MHC class I D-region cell surface antigen	**–8.9**	**2.1**	2.8	1.2
TCRβV8.2	T-cell receptor ß, variable 8.2	**–6.6**	**1.8**	–1.1*	2.0
Qb-1	MHC class I Q4 β-2-microglobulin (Qb-1)	**–6.4**	**1.2**	2.8*	0.9
H2-T10	Histocompatibility 2, T region locus 10	**–4.0**	**0.9**	0.4*	1.3
H2-T17	Histocompatibility 2, T region locus 17	**–3.9**	**0.4**	1.1*	0.1
H2-D1	Histocompatibility 2, D region locus 1	**–3.6**	**0.3**	2.5	0.8
H2	Histocompatibility 2, blastocyst	**–3.6**	**0.5**	2.0*	0.5
B2m	β-2 microglobulin	**–3.1**	**0.7**	1.8	0.7
TAPbp	TAP binding protein	**–2.7**	**0.3**	3.1*	0.4
TCRαV11.1d	T cell receptor α chain variable region, allele S1	**–2.4**	**0.6**	1.5*	0.1
Cytokines					
IL-1β	Interleukin 1ß	**–37.5**	**10.8**	0.0*	2.5
B94, TNFaip2	Tumor necrosis factor induced protein 2	**–32.6**	**6.8**	1.5*	3.1
IL-18bp, IGIFbp	Interleukin 18 binding protein	**–22.6**	**5.3**	–0.4*	1.3
TGF-β1	Transforming growth factor-ß1	**–23.7**	**6.9**	2.1*	0.6
IFN-β, TIMP	Interferon β, fibroblast	**–5.9**	**1.5**	1.1*	0.1
OPN, SPP1	Minopontin (osteopontin)	**–2.4**	**0.4**	1.2*	0.2
Chemokines					
MIP-1γ, CCL9/10, MRP-2, CCF18	Small inducible cytokine A9	**–63.5**	**10.9**	2.5*	0.8
MIG, CXCL9	Monokine induced by gamma interferon	**–59.1**	**10.8**	1.7*	0.2
IP-10, CXCL10, CRG-2	Macrophage interferon inducible protein 10	**–36.1**	**7.6**	1.7*	0.3
MCP-3, CCL7	Small inducible cytokine A7	**–18.7**	**5.2**	–0.5*	1.5
Cytokine receptors					
IL-4R	Interleukin 4 receptor (secreted form)	**–24.9**	**7.1**	1.9*	1.2
IL-3Rβ1, AIC2A	Interleukin 3 receptor, β chain 1	**–21.9**	**1.7**	–2.7*	1.7
IL-3Rβ2, AIC2B	Interleukin 3 receptor, β chain 2	**–19.1**	**3.4**	–1.9*	0.7
TNFR2, p75	p75 TNF receptor	**–4.5**	**1.3**	1.4*	0.2
IFNaR2b	Type I interferon receptor, IFNaR2b	**–3.3**	**0.5**	0.7*	1.5
CSF1R	Colony stimulating factor 1 receptor	**–3.1**	**0.1**	1.6*	0.3
LTβR	Lymphotoxin β receptor	**–3.1**	**0.8**	1.4*	0.7
IL-10Rβ	Interleukin 10 receptor, ß	**–2.9**	**0.8**	0.5*	1.3
Chemokine receptors					
CXCR-2	Chemokine (C-X-C) receptor 2	**–2.5**	**0.5**	1.8*	0.5

Adhesion molecules					
ITGβ5	Integrin ß-5	**–27.3**	**8.1**	10.5*	2.7
TGF-βi	Transforming growth factor, ß induced, 68 kDa	**–25.7**	**7.3**	2.1*	0.7
ITGαM	Cell surface glycoprotein Mac-1 α-chain	**–23.2**	**2.8**	1.9*	0.5
T-VCAM-1	Vascular cell adhesion molecule-1 truncated form T-VCAM-1 (VCAM-1)	**–4.9**	**1.1**	2.3	0.5
FN	Fibronectin	**–4.7**	**0.9**	2.6	0.9
Cyr61, CCN1	Insulin-like growth factor binding protein 10	**–4.3**	**1.2**	1.2*	2.2
VCAM-1	Vascular cell adhesion molecule 1	**–2.4**	**0.5**	0.2*	1.3
Cluster differentiation antigens					
CD68	CD68 antigen (macrosialin)	**–11.6**	**2.4**	1.6*	0.2
CD72	CD72 antigen	**–11.4**	**3.4**	0.3*	1.7
CD39	CD39 antigen	**–7.1**	**2.0**	–1.1*	0.1
CD86	CD86 antigen	**–2.5**	**0.2**	1.3*	0.2
CD38	CD38 antigen	**–2.4**	**0.4**	–1.3*	0.3
Apoptosis/death					
CASP8	Caspase-8	**–9.9**	**2.2**	0.2*	1.7
CASP11	Caspase-11	**–7.7**	**1.2**	2.2*	0.8
CTSH	Cathepsin H	**–7.4**	**1.0**	1.7	0.6
MPS-1	Macrophage proliferation-specific gene-1	**–6.9**	**1.5**	1.7	0.4
CASP1, ICE	Interleukin 1-β converting enzyme	**–5.4**	**1.3**	–0.1*	1.6
FAS	Fas antigen	**–3.3**	**0.9**	–1.4*	0.3
STFB	Cystatin B	**–3.0**	**0.6**	–1.1*	0.0
Signal transduction					
IIGP	Interferon-inducible GTPase	**–69.6**	**17.8**	–0.5*	1.5
TGTP	T-cell specific GTPase	**–68.6**	**10.9**	2.3*	0.59
IFI47, IRG-47	Interferon γ inducible protein, 47 kDa	**–58.3**	**16.1**	–0.2*	1.6
LCKPB1	Similar to human haematopoietic-specific protein 1	**–22.9**	**5.6**	0.7*	1.6
EMR1, F4/80	EGF-like module containing, mucin-like, hormone receptor-like sequence 1	**–19.6**	**1.6**	1.1*	2.5
PTPRC	Protein tyrosine phosphatase, receptor type, C	**–19.3**	**4.1**	1.9	0.7
IGTP	GTPase IGTP	**–16.1**	**3.3**	1.6*	0.3
IκBKE	IκK-i inducible IκB kinase	**–14.3**	**1.5**	–1.7*	0.5
LRG-47, IFI47	G-protein-like LRG-47	**–10.5**	**1.6**	1.4*	0.2
GTPI	GTPI protein	**–8.3**	**1.9**	1.4	0.2
TEP1	Telomerase associated protein 1	**–7.2**	**2.0**	–0.3*	1.6
DCIR, CLECSF6	Dendritic cell immunoreceptor	**–2.9**	**0.6**	–1.7*	0.2
MAPKAPK-2	MAP kinase-activated protein kinase 2	**–2.7**	**0.7**	2.8	0.5
LCK	Lck encoding lymphocyte-specific protein tyrosine kinase	**–2.6**	**0.8**	0.3*	1.3
FcgR1	Fc receptor, IgG, high affinity I	**–2.4**	**0.4**	1.3*	0.2
Transcription regulation					
mCDC46, MCMD5	mCDC46 protein	**–12.6**	**2.6**	1.9*	2.7
STAT6	Signal transducer and activator of transcription 6	**–10.5**	**3.2**	2.3*	1.7
STAT1	Signal transducer and activator of transcription 1	**–6.7**	**0.9**	–0.2*	1.5
IRF1	Interferon regulatory factor 1	**–5.9**	**1.6**	1.3*	0.1
TGIF	mTGIF protein	**–5.5**	**0.3**	0.4*	1.4
ICSbp	Interferon consensus sequence binding protein	**–4.4**	**1.0**	1.3*	0.1
APOBEC1	Apolipoprotein B editing complex 1	**–3.9**	**1.0**	1.5*	0.1
MIRF7	Interferon regulatory factor 7	**–3.0**	**0.8**	1.3*	0.2
ISGF3γ	Interferon dependent positive acting transcription factor 3 γ	**–2.9**	**0.6**	0.4*	1.3
Acute phase/complement activation					
SAA	Serum amyloid A	**–176.9**	**50.7**	4.9	3.1
SPI2-1, EB1, SPI2	Spi2 proteinase inhibitor	**–52.6**	**12.3**	–1.8*	2.7
H2-Bf	Histocompatibility 2, complement component factor B	**–5.5**	**1.2**	3.5*	0.8
C3aR1	Complement component 3a receptor	**–5.4**	**1.5**	1.3*	0.3
C1qβ	Complement C1q β chain	**–4.7**	**0.9**	2.4	0.7
C1qa	Complement component 1, q subcomponent, α polypeptide	**–4.2**	**0.9**	2.6	0.6
C1qc	Complement component 1, q subcomponent, c polypeptide	**–4.1**	**1.2**	2.1	0.7
C3	Complement component C3 , α and β subunits	**–3.5**	**0.6**	1.6	0.5

CNS-related genes					
VAMP8	Endobrevin	**–21.9**	**3.7**	32.3*	4.5
NINJ1	Ninjurin	**–13.5**	**4.0**	–1.1*	2.5
Other genes					
MCL, MPCL, CLECSF8	C-type lectin	**–33.8**	**5.5**	–3.2*	1.1
TCIRG1, ATP6i, OC116, TIRC7	T-cell, immune regulator 1	**–32.2**	**7.7**	49.9*	16.3
PIRA3	Paired-Ig-like receptor A3	**–32.1**	**5.4**	0.9*	3.0
IRG1	Immunoresponsive 1	**–18.6**	**3.7**	38.3*	16.7
GP-39, Chi3l1	Glycoprotein 39	**–9.3**	**2.2**	1.5*	0.2
CLIC1, CLC-1, NCC27	Chloride intracellular channel 1	**–6.2**	**1.9**	8.2	2.6
FGL2	Fibrinogen-like protein 2	**–5.7**	**0.9**	–0.4*	0.3
ADRP	Adipose differentiation-related protein	**–5.3**	**0.9**	1.2*	0.1
E3, LAPTM5	Retinoic acid-inducible E3 protein	**–3.9**	**0.8**	1.8	0.4
XANX-4, ANXA4	Annexin IV	**–3.2**	**0.3**	1.3*	0.1
LGALS9	β-galactoside binding lectin	**–2.8**	**0.9**	1.4*	0.2
FTL1	Ferritin light chain 1	**–2.8**	**0.3**	1.5*	0.2

* Expression was at or below background level in SC from naïve mice.

Table 2. Up-regulation of adhesion and signal transduction molecules

Name	Gene description	**Estrogen over EAE**		Estrogen over naive	
		fold change	**SD**	fold change	SD
Signal transduction					
FEX	Orphan G protein-coupled receptor FEX	**3.3**	**0.9**	1.8*	0.4
GPR26	G-protein coupled receptor	**3.3**	**0.9**	1.1*	0.1
Transcription regulation					
RNPS1	Ribonucleic acid binding protein S1	**9.9**	**2.2**	–0.4*	1.5
CNS-related genes					
BCAN	Brevican	**6.1**	**0.2**	0.7*	1.8
Other genes					
ALAS2	Aminolevulinic acid synthase 2, erythroid	**6.6**	**0.3**	2.8	1.3
Slc27a2	Fatty acid transport protein	**3.0**	**0.7**	–0.1*	2.1

* Expression was at or below background level in SC from naïve mice.

Results

Using the Affymetrix microarray technology, we obtained transcriptional profiles from mRNA isolated from the spinal cords of two experimental groups of double Tg females immunized with MBP-Ac1-11 peptide: 1) mice displaying severe symptoms of EAE (score 5) implanted with a control pellet (which did not affect the course of EAE; our unpublished data) and 2) mice with no signs of EAE (score 0) implanted with a 2.5 mg pellet of E2 (physiological equivalence of 20–50% of pregnancy levels) on the back 7 days before the immunization. Unmanipulated mice served as a control. The assay was performed in triplicate for each group except for the group of unmanipulated mice. Spinal cords from two unmanipulated mice were pooled and only one microarray chip was used to minimize the costs of assay. The fold change (FC) presented for each gene in Tables 1 and 2 is the mean value from three separate experiments with SD less than 30% in estrogen-treated mice over the EAE mice. The fold change for each particular gene in the category estrogen-treated over unmanipulated is also presented in Tables 1 and 2. However, the values marked with an asterisk (*) indicate that the expression in the unmanipulated mice was at or below background levels. The same assembly of 12,000 genes on each array (MG_U47A) was used as in our previous study. Similar to E2 affects on splenocytes, estrogen therapy altered expression of only about 10% of all genes present on the chip. However, unlike splenocytes, the great majority of effected genes in the CNS tissue were down-regulated – 302 genes vs only 13 genes that were up-regulated. From this larger set, we grouped 119 genes

of interest according to their function or nomenclature. Genes which did not fit these categories are presented as "other genes".

Estrogen treatment provides complete protection from EAE in DTg female mice

Clinical EAE data for mice that developed severe symptoms of disease and for estrogen-protected animals were presented previously[20]. Females implanted with 2.5 mg pellets of E2 did not develop any signs of disease 14–16 days after immunization with MBP-Ac1--11 peptide/CFA. None of the estradiol-treated mice developed a score other than 0. In contrast, control mice not receiving E2 displayed severe hind limb weakness and paraplegia, reaching a score of 5–6. Serum E2 levels in mice implanted with estrogen were between 1000– 2000 pg/ml.

Genes with decreased expression

More than 95% of genes presented in this work displayed down-regulation in the E2-treated group with no EAE versus the EAE control group with severe EAE, and are presented in Table 1. We grouped down--regulated genes into several categories. The most numerous group includes genes involved in "Antigen presentation/processing". One of these genes, MHC class II antigen A (H2Aa), displayed the highest (–203.6) FC out of all found genes. Four other genes in this group also displayed more than a 20 FC: proteosome B type 9 (LMP2/PSMB9, –70.5), IFN-γ inducible protein 30 (GILT/IP-30, – 47.3), MHC class II locus Mβ1 (H2-DMβ1, –35), and MHC class I antigen Eβ (H2--Eβ1, –27.7). Among "Cytokines", four genes showed more than a 20 FC, including: IL-1β (–37.5), TNF-aip2 (–32.6), IL-18bp/IGIFbp (– 22.6) and TGF-β1 (– 23.7). Strong down-regulation was observed in "Chemokines" with – 63.5 FC for MIP-Iγ, – 59.1 FC for MIG and – 36.1 FC for IP-10. It is noteworthy that only one "Chemokine receptor", CXCR-2, was found to be effected by estrogen treatment and was only down-regulated by 2.5 fold. However, 8 "Cytokine receptor" genes were down--regulated, including IL-4R (secreted form) (– 24.9FC), IL-3Rβ1 and IL-3Rβ2 (– 21.9 and – 19.1 respectively), p75 TNFR2 (– 4.5) and others. The groups of genes related to "Cluster differentiation antigens", "Apoptosis/death", and "Transcription regulation" displayed only moderate fold changes between 2.4 and 12.6. In contrast, "Adhesion Molecules" and "Signal transduction" – related genes showed relatively strong down--regulated expression as presented by ITGβ5 (– 27.3 FC), TGF-βi (–25.7 FC), ITGαM (–23.2 FC) and IIGP (–69.6 FC), TGTP (–68.6 FC), IFI47/IRG-47 (–58.3 FC), and LCKPB1 (– 22.9 FC), respectively. Also, two genes classified in the "Acute phase/complement" group showed strong transcriptional changes: SAA (–176.9 FC) and SPI-2-1 (– 52.6 FC). Of note, we found some "CNS tissue-related" genes to be inhibited in estrogen-treated mice, such as VAMP8 (–21.9 FC) and NINJ1 (–13.5 FC). Among "Other genes", the highest reduction in expression was displayed by MCL (–33.8 FC), TCIRG1 (–32.3 FC) and PIRA3 (– 32.1 FC).

Genes with increased expression

Only 6 genes demonstrated significant up-regulation in expression, presented in Table 2. The highest FC was found for gene RNPS1 (+9.9 FC) classified as "Transcription regulation" related. Two other genes: BCAN classified as "CNS-related", and ALAS2, as "Other genes", showed +6.1 FC and +6.6 FC, respectively. Three other genes displayed FC between 3 and 3.3.

Discussion

The results presented above demonstrate conclusively that E2 treatment of EAE results in a strong and pervasive down-regulation of inflammation-related genes in the SC. The transcriptional gene pattern presented here for SC tissue was markedly different from that published previously for spleen cells. In fact, only two of the genes in the SC were also significantly changed in the same direction in the spleen: ALAS2 (aminolevulinic acid synthase 2: SC, +6.6 FC; spleen, +30.0 FC), which catalyzes the first step of the heme biosynthetic pathway found also in the brain[8], and DCIR (dendritic cell immunoreceptor: SC, – 2.9 FC; spleen: –4.0 FC), found to have a negative regulatory function on antigen-presenting cells[16]. Moreover, the effect of hormonal therapy on CNS gene expression was stronger than that found in the spleen, with 27 of the genes displaying >20 fold changes.

The most affected gene, H2Aa, down-regulated by 203 fold, is the sole restriction element for the encephalitogenic MBP-Ac1-11 peptide in this strain of double Tg mouse, and is obviously required for EAE induction. The majority of genes in this general category of "Antigen presentation/processing" were MHC genes associated with the H2 locus, which has clearly been linked to EAE susceptibility[10]. Four other genes within this group displayed strong fold changes:

proteosome β type 9 (LMP2/PSMB9; –70.5 FC), which is a TNF-α- and IFN-γ-dependent provider of peptide ligands for MHC class I[12]; IFN-γ-inducible protein 30 (GILT/IP-30; – 47.3 FC), required for antigen processing, GILT-free mice being defective in this function[18]; MHC class II locus Mβ1 (H2-DMβ1; – 35 FC) an MHC class II-like molecule with a crucial role in antigen procession and presentation and associated with the development of autoimmune diseases[32]; and MHC class II antigen Eβ (H2-Eβ1; – 27.7 FC). Inhibition properties of estrogens on antigen presentation have been reported previously[34], and in combination with the immunosuppressant cyclosporine, estrogen abolishes MHC class II expression in allografts[25]. Besides genes associated with class II MHC, there were also differences in the gene expressions of MHC class I molecules, including MHC class I D-region cell surface antigen (D2d; –8.9 FC); MHC class I Q4 β2-microglobulin (Qb-1; – 6.4 FC), reported to be secreted from activated lymphocytes[7]; and β2-microglobulin (β2-m; – 3.1 FC). It is noteworthy that the BV8S2 transgene specific for MBP-Ac1-11 was also down-regulated 8.9 fold.

The strong down-regulation in E2-treated mice of genes for MHC class I and II expression, β2-microglobulin, and complement components C3 (C3; – 3.5 FC), thought to play a pivotal role in the degeneration of oligodendrocytes[22], and C1q (3 C1q subunits; FC from – 4.1 to – 4.7), which contributes to the development of neurological diseases including EAE[9], might suggest that E2 interferes with IFN-γ production. Although we did not observe changes in the IFN-γ gene, we did find changes in several IFN-γ-related genes, including interferon regulatory factor 1 (IRF1; –5.9 FC), the absence of which decreased production of IFN-γ[27], and interferon consensus sequence binding protein (ICSbp; – 4.4 FC), a transcription factor of the IFN regulatory factor family[28]. Moreover, we observed down-regulation of several IFN-γ-inducible genes, including interferon-inducible protein 10 (IP-10; – 63.5 FC) and monokine induced by IFN-γ (MIG; – 59.1 FC), both produced by macrophages and reactive astrocytes in actively demyelinating MS lesions[26]; IL-18 binding protein (IL-18bp/IGIFbp; – 22.6 FC), strongly induced by IFN-γ and other Th1 cytokines[31]; IFN-γ inducible protein 30 (IFI30/IP30/GILT; – 47.3 FC); four highly down-regulated interferon-inducible GTPases: IIGP protein (– 69.6 FC), shown to be related to neuronal loss in scrapie-infected brain tissue[24]; TGTP (– 68.6 FC), which may have an important function in T cell development and/or T cell activation[4]; IRG-47 (–58.3FC), expressed in B cells[11]; and GTPase IGTP (–16.1 FC), expressed in macrophages[29]; as well as other genes involved in signal transduction and transcription regulation listed in Table 1.

Besides IFN-γ-dependent genes, there were also TNF-α-related and -regulated genes, including IκB kinase (IKBKE; –14.3 FC), which plays a key role in the regulation of nuclear factor κB and was up-regulated by minimal doses of TNF-α[2]; and TFN – induced protein 2 (B94; –32.6 FC), found on mature peripheral blood monocytes[35]. Moreover, genes for the p75 TNF receptor (p75TNFR2; –4.5FC), implicated as a susceptibility gene in linkage analysis in MS patients[6], and lymphotoxin-β receptor (LT-β; – 3.1 FC) were also down-regulated in E2-treated mice. In contrast to splenocytes, where E2 treatment strongly down-regulated the expression of TNF-α, no direct change in the TNF-α gene was observed in SC tissue. The most likely explanation is that E2 treatment systemically inhibited TNF-α in inflammatory T cells and monocytes/macrophages, which were prevented from infiltrating SC tissue by other E2-affected mechanisms. Another consideration is that in inflamed SC tissue, especially at the acute phase of disease, E2 might down-regulate CNS genes that contribute to EAE severity. CNS-associated genes that were strongly down-regulated might include Endobrevin (VAMP8, – 21.9 FC vs. EAE SC; – 32.3 FC vs. naïve SC), a synaptic protein with a structure similar to VAMP2 that was found in MS patients[17]; and Ninjurin (–13.5 FC), also called nerve injury-induced protein that is up-regulated after axotomy in neurons and in Schwann cells[1]. The single CNS-associated gene that was up-regulated was Brevican (+6.1 FC), an ECM molecule influencing astroglial motility[30].

The failure to detect so called "classical" proinflammatory cytokines/chemokines was also reported by Ibrahim et al.[14], who studied the gene expression profile of CNS in C57Bl/6 mice. Interestingly, one classical cytokine inhibited by E2 was IL-1β, (– 37.5 FC), a critical cytokine known to contribute to the initiation and progression of EAE[33]. Importantly, several altered genes identified by Ibrahim were confirmed by our study, for example genes involved in the regulation of IFN-γ transcription and chemokines such as IP-10 (– 36.1 FC) and MIP-1γ (– 63.5 FC), chemokines produced by DC that mediate the migration of both activated and non-activated $CD4^+$ and $CD8^+$ T cells[21].

Other E2-sensitive genes also deserve mention: CD68 (– 11.6 FC) (microsialin), which showed positive staining in MS patients and can contribute to foam cell formation in atherosclerotic lesions[23]; caspase-8 (–9.9 FC), the activity of which was inhibited *in vitro* by E2-treated neuronal extracts[36]; caspase-11 (– 7.7 FC), which plays

a crucial role in oligodendrocyte death and pathogenesis in EAE[13]; and osteopontin (– 2.4 FC), as osteopontin knockout mice were resistant to progressive EAE as shown by CHABAS et al.[5]

In conclusion: 1) the gene expression profile found in the spinal cord was markedly different from that found in splenocytes; 2) the strong down-regulation of the transgenic T cell receptor gene TCRβV8.2 (–6.6 FC) and macrophage genes (eg. MAC-1 α-chain, – 23.2 FC; and F4/80, –19.6 FC) would suggest that a major effect of estrogen is to inhibit migration of inflammatory mononuclear cells into the target organ, producing a net effect very similar to that of spinal cord tissue from unmanipulated mice; 3) the majority of E2-sensitive genes that were down-regulated in the SC were TNF-α/IFN-γ related or regulated, suggesting hormone-dependent regulation of these cytokines; 4) a number of SC genes that were differentially affected by E2 treatment might be implicated as contributing to EAE susceptibility; 5) based on this broad scan, several other E2-sensitive pathways may be implicated, such as the apoptotic cascade and signal transduction*. Future studies will focus on validation of the role of EAE susceptibility and protective genes that potentially could be evaluated for their contribution to MS by RT-PCR.

Acknowledgment. The authors wish to thank Dr. Chris Harrington, Brian Tompkins, and Kristina Vartanian of the Affymetrix Microarray Core of the OHSU Gene Microarray Shared Resource for expert assistance with sample and data processing, and Ms Eva Niehaus for assistance with the manuscript.

References

1. ARAKI T. and MILBRANDT J. (1996): Ninjurin, a novel adhesion molecule, is induced by nerve injury and promotes axonal growth. Neuron, **17**, 353– 361.
2. AUPPERLE K. R., YAMANISHI Y., BENNETT B. L., MERCURIO F., BOYLE D. L. and FIRESTEIN G. S. (2001): Expression and regulation of inducible IκB kinase (IKK-i) in human fibroblast-like synoviocytes. Cell. Immunol., **214**, 54– 59.
3. BEBO B. F. JR., FYFE-JOHNSON A., ADLARD K., BEAM A. G., VANDENBARK A. A. and OFFNER H. (2001): Low-dose estrogen therapy ameliorates experimental autoimmune encephalomyelitis in two different inbred mouse strains. J. Immunol., **166**, 2080– 2089.
4. CARLOW D. A., MARTH J., CLARK-LEWIS I. and TEH H. S. (1995): Isolation of a gene encoding a developmentally regulated T cell-specific protein with a guanine nucleotide triphosphate-binding motif. J. Immunol., **154**, 1724–1734.
5. CHABAS D., BARANZINI S. E., MITCHELL D., BERNARD C. C., RITTLING S. R., DENHARDT D. T., SOBEL R. A., LOCK C., KARPUJ M., PEDOTTI R., HELLER R., OKSENBERG J. R. and STEINMAN L. (2001): The influence of the proinflammatory cytokine, osteopontin, on autoimmune demyelinating disease. Science, **294**, 1731– 1735.
6. CROXFORD J. L., O'NEILL J. K. and BAKER D. (1997): Polygenic control of experimental allergic encephalomyelitis in Biozzi ABH and BALB/c mice. J. Neuroimmunol., **74**, 205–211.
7. DAY P. M. and FRELINGER J. A. (1991): Differential expression of Q4 proteins (Qb-1) in fibroblasts and lymphocytes. J. Immunol., **147**, 3427– 3433.
8. DE MATTEIS F., ZETTERLUND P. and WETTERBERG L. (1981): Brain 5-aminolaevulinate synthase. Developmental aspects and evidence for regulatory role. Biochem. J., **196**, 811– 817.
9. DIETZSCHOLD B., SCHWAEBLE W., SCHAFER M. K., HOOPER D. C., ZEHNG Y. M., PETRY F., SHENG H., FINK T., LOOS M., KOPROWSKI H. et al. (1995): Expression of C1q, a subcomponent of the rat complement system, is dramatically enhanced in brains of rats with either Borna disease or experimental allergic encephalomyelitis. J. Neurol. Sci., **130**, 11–16.
10. ENCINAS J. A., WEINER H. L. and KUCHROO V. K. (1996): Inheritance of susceptibility to experimental autoimmune encephalomyelitis. J. Neurosci. Res., **45**, 655–669.
11. GILLY M. and WALL R. (1992): The IRG-47 gene is IFN-γ induced in B cells and encodes a protein with GTP-binding motifs. J. Immunol., **148**, 3275–3281.
12. GROETTRUP M., VAN DEN BROEK M., SCHWARZ K., MACAGNO A., KHAN S., DE GIULI R. and SCHMIDTKE G. (2001): Structural plasticity of the proteosome and its function in antigen processing. Crit. Rev. Immunol., **21**, 339– 358.
13. HISAHARA S., YUAN J., MOMOI T., OKANO H. and MIURA M. (2001): Caspase-11 mediates oligodendrocyte cell death and pathogenesis of autoimmune-mediated demyelination. J. Exp. Med., **193**, 111– 122.
14. IBRAHIM S. M., MIX E., BOTTCHER T., KOCZAN D., GOLD R., ROLFS A. and THIESEN H. J. (2001): Gene expression profiling of the nervous system in murine experimental autoimmune encephalomyelitis. Brain, **124**, 1927– 1938.
15. ITO A., BEBO B. F. JR., MATEJUK A., ZAMORA A., SILVERMAN M., FYFE-JOHNSON A. and OFFNER H. (2001): Estrogen treatment down-regulates TNFα production and reduces the severity of experimental autoimmune encephalomyelitis in cytokine knockout mice. J. Immunol., **167**, 542–552.
16. KANAZAWA N., OKAZAKI T., NISHIMURA H., TASHIRO K., INABA K. and MIYACHI Y. (2002): DCIR acts as an inhibitory receptor depending on its immunoreceptor tyrosine-based inhibitory motif. J. Invest. Dermatol., **118**, 261–266.
17. LOCK C., HERMANS G., PEDOTTI R., BRENDOLAN A., SCHADT E., GARREN H., LANGER-GOULD A., STROBER S., CANNELLA B., ALLARD J., KLONOWSKI P., AUSTIN A., LAD N., KAMINSKI N., GALLI S. J., OKSENBERG J. R., RAINE C. S., HELLER R. and STEINMAN L. (2002): Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. Nat. Med., **8**, 500–508.
18. MARIC M., ARUNACHALAM B., PHAN U. T., DONG C., GARRETT W. S., CANNON K. S., ALFONSO C., KARLSSON L., FLAVELL

* Noteworthy: the results obtained by Affymatrix for two genes presented in Table 1 were confirmed by RPA technique: IP-10 showed 30 fold and TGFβ1 showed 4.5 fold decrease (unpublished data).

R. A. and CRESSWELL P. (2001): Defective antigen processing in GILT-free mice. Science, **294**, 1361–1365.
19. MATEJUK A., ADLARD K., ZAMORA A., SILVERMAN M., VANDENBARK A. A. and OFFNER H. (2001): 17-beta estradiol inhibits cytokine, chemokine, and chemokine receptor mRNA expression in the central nervous system of female mice with experimental autoimmune encephalomyelitis. J. Neurosci. Res., **65**, 529–542.
20. MATEJUK A., DWYER J., ZAMORA A., VANDENBARK A. A. and OFFNER H. (2002): Evaluation of the effects of 17-beta estradiol on gene expression in experimental autoimmune encephalomyelitis using DNA microarray. Endocrinology, **143**, 313–319.
21. MOHAMADZADEH M., POLTORAK A. N., BERGSTRESSOR P. R., BEUTLER B. and TAKASHIMA A. (1996): Dendritic cells produce macrophage inflammatory protein-1 γ, a new member of the CC chemokine family. J. Immunol., **156**, 3102–3106.
22. NATAF S., CARROLL S. L., WETSEL R. A., SZALAI A. J. and BARNUM S. R. (2000): Attenuation of experimental autoimmune demyelination in complement-deficient mice. J. Immunol., **165**, 5867–5873.
23. RAMPRASAD M. P., TERPSTRA V., KONDRATENKO N., QUEHENBERGER O. and STEINBERG D. (1996): Cell surface expression of mouse macrosialin and human CD68 and their role as macrophage receptors for oxidized low density lipoprotein. Proc. Natl. Acad. Sci. USA, **93**, 14833–14838.
24. RIEMER C., QUECK I., SIMON D., KURTH R. and BAIER M. (2000): Identification of unregulated genes in scrapie-infected brain tissue. J. Virol., **74**, 10245–10248.
25. SAITO S., FOEGH M. L., MOTOMURA N., LOU H., KENT K. and RAMWELL P. W. (1998): Estradiol inhibits allograft-inducible major histocompatibility complex class II antigen expression and transplant arteriosclerosis in the absence of immunosuppression. Transplantation, **66**, 1424–1431.
26. SIMPSON J. E., NEWCOMBE J., CUZNER M. L. and WOODROOFE M. N. (2000): Expression of the interferon-γ-inducible chemokines IP-10 and Mig and their receptor, CXCR3, in multiple sclerosis lesions. Neuropathol. Appl. Neurobiol., **26**, 133–142.
27. TADA Y., HO A., MATSUYAMA T. and MAK T. W. (1997): Reduced incidence and severity of antigen-induced autoimmune diseases in mice lacking interferon regulatory factor-1. J. Exp. Med., **185**, 231–238.
28. TAMURA T. and OZATO K. (2002): ICSBP/IRF-8: its regulatory roles in the development of myeloid cells. J. Interferon Cytokine Res., **22**, 145–152.
29. TAYLOR G. A., JEFFERS M., LARGAESPADA D. A., JENKINS N. A., COPELAND N. G. and WOUDE G. F. (1996) Identification of a novel GTPase, the inducibly expressed GTPase, that accumulates in response to interferon γ. J. Biol. Chem., **271**, 20399–20405.
30. THON N., HAAS C. A., RAUCH U., MERTEN T., FASSLER R., FROTSCHER M. and DELLER T. (2000): The chondroitin sulphate proteoglycan brevican is up regulated by astrocytes after entorhinal cortex lesions in adult rats. Eur. J. Neurosci., **12**, 2547–2558.
31. VEENSTRA K. G., JONAK Z. L., TRULLI S. and GOLLOB J. A. (2002): IL-12 induces monocyte IL-18 binding protein expression via IFN-γ. J. Immunol., **168**, 2282–2287.
32. WALTER W., SCHEUER C., LOOS M., REICHERT T. E. and MAEURER M. J. (2001): H2-Mβ1 and H2-Mβ2 heterodimers equally promote clip removal in I-A(q) molecules from autoimmune-prone DBA/1 mice. J. Biol. Chem., **276**, 11086–11091.
33. WIEMANN B., VAN G. Y., DANILENKO D. M., YAN Q., MATHESON C., MUNYAKAZI L., OGENSTAD S. and STARNES C. O. (1998): Combined treatment of acute EAE in Lewis rats with TNF-binding protein and interleukin-1 receptor antagonist. Exp. Neurol., **149**, 455–463.
34. WIRA C. R., ROSSOLL R. M. and KAUSHIC C. (2000): Antigen--presenting cells in the female reproductive tract: influence of estradiol on antigen presentation by vaginal cells. Endocrinology, **141**, 2877–2885.
35. WOLF F. W., SARMA V., SELDIN M., DRAKE S., SUCHARD S. J., SHAO H., O'SHEA K. S. and DIXIT V. M. (1994): B94, a primary response gene inducible by tumor necrosis factor-α, is expressed in developing hematopoietic tissues and the sperm acrosome. J. Biol. Chem., **269**, 3633–3640.
36. ZHANG Y., TOUNEKTI O., AKERMAN B., GOODYER C. G. and LEBLANC A. (2001): 17β-estradiol induces an inhibitor of active caspases. J. Neurosci., **21**, RC176.

Received in December 2002
Accepted in January 2003