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Identification of estrogen-responsive genes in the GH3 cell line by cDNA microarray analysis

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Abstract

To identify estrogen-responsive genes in somatolactotrophic cells of the pituitary gland, a rat pituitary cell line GH3 was subjected to cDNA microarray analysis. GH3 cells respond to estrogen by growth as well as prolactin synthesis. RNAs extracted from GH3 cells treated with 17β-estradiol (E2) at 10^{-9} M for 24 h were compared with the control samples. The effect of an antiestrogen ICI182780 was also examined. The array analysis indicated 26 genes to be up-regulated and only seven genes down-regulated by E2. Fourteen genes were further examined by real-time RT-PCR quantification and 10 were confirmed to be regulated by the hormone in a dose-dependent manner. Expression and regulation of these genes were then examined in the anterior pituitary glands of female F344 rats ovariectomized and/or treated with E2 and 8 out of 10 were again found to be up-regulated. Interestingly, two of the most estrogen-responsive genes in GH3 cells were strongly dependent on E2 in vivo. #1 was identified as calbindin-D9k mRNA, with 80- and 118-fold induction over the ovariectomized controls at 3 and 24 h, respectively, after E2 administration. #2 was found to be parvalbumin mRNA, with 30-fold increase at 24 h. Third was c-*myc* mRNA, with 4.5 times induction at 24 h. The levels were maintained after one month of chronic E2 treatment. Identification of these estrogen-responsive genes should contribute to understating of estrogen actions in the pituitary gland.
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Keywords: Estrogen-responsive genes; cDNA microarray; Pituitary; GH3; Rats

1. Introduction

Estrogen regulates multiple functions in different cell types in the anterior pituitary gland [1–3]. In the somatolactotrophs (GH/prolactin cells), it is well documented that estrogen activates prolactin mRNA transcription through the estrogen-responsive element (ERE) located in the 5′-upstream regulatory region [4,5]. The storage and release of prolactin are also regulated by estrogen [6]. In addition to hormone production, estrogen promotes cell proliferation in somatolactotrophs, which is prominent in the rat case [7–9]. Although estrogen-responsive expression of a series of genes must be involved in these biological functions of the pituitary cells, only a few have so far been reported to be regulated by estrogen [2].

GH3 is a widely used rat pituitary somatolactotrophic cell line, originally isolated from the MtT/W5 pituitary tumor, whose growth and prolactin synthesis are stimulated by estrogen [10,11]. There is a variation in the estrogen-responsiveness of this cell line reported in the literature [5,12–15], but the cells obtained from the Health Science Research Resources Bank in Osaka, Japan, display high sensitivity with regard to induction of cell proliferation. In the present study, we performed a gene expression analysis of estrogen action in GH3 cells using the cDNA microarray technique and found many of the identified estrogen-responsive genes to also be similarly regulated in vivo in the anterior pituitary in F344 rats.

2. Materials and methods

2.1. Chemicals

17β-estradiol (E2) was purchased from Sigma Chemicals, St. Louis, MO, USA and ICI182780 was obtained from Tocris Cookson Ltd., Bristol, UK. Each was dissolved in

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ethanol to give stock solutions. Actinomycin D and cycloheximide were purchased from Wakojunyaku KK, Osaka, Japan.

2.2. Cell culture

The pituitary cell line GH3 was obtained from the Health Science Research Resources Bank (Osaka, Japan) and maintained in DME/F12 mixed medium (Sigma Chemical Co.) containing penicillin and streptomycin with 10% horse serum (HS, Gibco/Invitrogen Corp., Carlsbad, CA, USA) and 2.5% fetal bovine serum (FBS, Gibco/Invitrogen). Before estrogen treatment, cells were maintained for a week in phenol red-free medium (Sigma Chemicals) containing the same antibiotics along with dextran-charcoal-treated serum. For cell growth assays, GH3 cells were seeded in 24-well plates at 1×10^4 cells/well, and hormones were added the next day. Growth was measured after five days by means of a modified MTT assay with WST-1 (Dojindo Chemicals, Kumamoto, Japan). For microarray analysis, 3 $\times 10^6$ GH3 cells were seeded in 90 mm dishes and treated with E2 at 10^{-9} M and/or ICI at 10^{-7} M and harvested after 24 h treatment. Cells were harvested after addition of Isogen (Wakojunyaku). For mRNA quantification, cells were treated with E2 at 10^{-12} to 10^{-9} M and/or ICI182780 at 10^{-7} M. After the indicated period of time, cells were harvested with cell lysis buffer supplied with an SV-total RNA isolation kit (Promega Co., Madison, WI, USA).

2.3. Animals

Animal experiments were conducted under the guidelines of the 'A Guide for the Care and Use of Laboratory Animals of Hiroshima University'. Female F344 rats were purchased at four weeks of age from Charles River Japan Co. (Kanagawa, Japan). They were maintained with free access to basal diet and tap water. All animals except the intact control underwent surgical ovariectomy upon receipt and implanted with pellets containing 10 mg of E2 subcutaneously as described previously [16]. Animals were sacrificed under ether anesthesia after 3, 8, 24 and 48 h in the short-term experiment. Treatment was extended between 7 and 30 days for the long-term experiment. The pituitary gland and the uterus of each rat were weighed and immediately frozen in liquid nitrogen and stored at −80 °C.

2.4. The GeneChip analysis

Total RNAs were extracted with Isogen, a premixed RNA isolation reagent, based on the acid guanidium thiocyanate–phenol–chloroform extraction method. The supplied protocol was followed.

First-strand cDNA was synthesized by incubating 5 μg of total RNAs with 200 U SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA), 100 pmol T7-(dT)24 primer [5′-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT)24-3′], 1 × first-strand buffer (50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM DTT) and 0.5 mM dNTPs at 42 °C for 1 h. Second-strand synthesis was performed by incubating the first-strand cDNAs with 10 U *E. coli* ligase (Invitrogen), 40 U DNA polymerase I (Invitrogen), 2 U RNase H (Invitrogen), 1× reaction buffer (18.8 mM Tris–HCl pH 8.3, 90.6 mM KCl, 4.6 mM $MgCl_2$, 3.8 mM DTT, 0.15 mM NAD, 10 mM $(NH_4)_2SO_4$) and 0.2 mM dNTPs at 16 °C for 2 h. Ten units of T4 DNA polymerase (Invitrogen) were then added, and the reaction was allowed to continue for another 5 min at 16 °C. After phenol–chloroform extraction and ethanol precipitation, the double-stranded cDNA was resuspended in 12 μl DEPC-treated dH_2O. Labeling of the dsDNA was achieved by in vitro transcription using a BioArray HighYield RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY). Briefly, the dsDNA was mixed with 1× HY reaction buffer, 1× biotin labeled ribonucleotides (NTPs with Bio-UTP and Bio-CTP), 1× DTT, 1× RNase inhibitor mix and 1× T7 RNA polymerase. The mixture was incubated at 37 °C for 4 h. The labeled cRNA was then purified using a RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purified cRNA was fragmented in 1 × fragmentation buffer (40 mM acetate, 100 mM KOAc, 30 mM MgOAc) at 94 °C for 35 min. For hybridization with the GeneChip Rat Genome U34A (Affymetrix), 15 μg fragmented cRNA probe was incubated with 50 pM control oligonucleotide B2, 1× eukaryotic hybridization control (1.5 pM BioB, 5 pM BioC, 25 pM BioD and 100 pM Cre), 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA and 1× manufacturer-recommended hybridization buffer in a 45 °C rotisserie oven for 16 h. Washing and staining were performed with a GeneChip Fluidic Station (Affymetrix) using the appropriate antibody amplification washing and staining protocol. The phycoerythrin-stained arrays were scanned as digital image files and scanned data were analyzed with GeneChip software (Affymetrix) [17].

2.5. Quantification of mRNAs by real-time RT-PCR

RNA preparation was carried out with an SV-total RNA isolation kit. One microgram of total RNA was reverse-transcribed with 200 U of MMLV-RT (Invitrogen) and 2.5 pmol of oligo-dT primer (Invitrogen) in 25 μl buffer containing 1 mM dNTP, 100 mM Tris–HCl (pH 8.3), 150 mM KCl, 6 mM $MgCl_2$, 60 mM dithiothreitol and 5 U/μl RNasin with incubation at 37 °C for 60 min.

The real-time PCR method with a QuantiTect Sybr Green PCR kit (Qiagen) and an ABI Prism 7700 (The Perkin–Elmer Co) was employed for quantitative measurement for following the supplied protocol [18]. Specific primer sets with a T_m of about 59 °C were designed for each mRNA selected from the microarray analysis (Table 1). The PCR conditions were a 15 min of initial activation step followed by 45 cycles of 15 s at 94 °C, 30 s at 50 °C and 60 s at

Table 1
Nucleotide sequences of primers for quantitative real-time PCR

Gene	GenBank accession#	Forward	Reverse
#1	K00994	AACCAGCTGTCCAAGGAGGA	CTTCTCCATCATCGTTCTTATCCA
#2	AI175539	TTTCTTCAGGCCCACCATCT	TTGCAGGATGTCGATGACAGA
#3	AI014135	GAACCAATTCTCCTAGCACAAGTG	CACGCCTGTGTTGGGCTAA
#4	AI178971	GGTGTGAAATCCCCAGGGT	CCCTGTCCACTCTGAGCGAC
#5	S81478	GATCAACGTCTCGGCCAATT	GCACAAACACCCTTCCTCCA
#6	D26393	GATTTCTAGGCGGTTCCGGA	ACTCGGAGCACACGGAAGTT
#7	AI230712	TGGCAGAAAAATCAATCCAGC	AAAGCCAGCCCCAAATCAC
#8	AF081366	CATCTGGACAACTGTGCTGGA	GGCACCACACATGAAGGAATT
#9	Y00396	CCGAGCTACTTGGAGGAGACA	AGGCCAGCTTCTCGGAGAC
#10	U02553	GATCAACGTCTCGGCCAATT	GCACAAACACCCTTCCTCCA
#14	U24175	CAGTGGATCGAGAGCCAGC	TGCCCCAGCTTGATCTTCAG
#15	D13623	ACCAAGACCGGTAGCAAGGG	GAAATCCGACGGAAGAGTGC
#21	AA892522	CCTTCGACTCAGCCACAAAAA	ACAGGGTCTTACCCTGCCTTC
#22	L16922	AGCCAGAGCCCACAATATGG	GCAATCATTTCTTCCGGCAC
G3PDH	AB017801	TGAAGGTCGGTGTGAACGGATTTG	TGATGGCATGGACTGTGGTCATGA

72 °C. Prior to the quantitative analysis, PCR products were prepared separately and purified by gel electrophoresis. The fragments extracted from the gel were used as standards for quantification. The DNA sequences were confirmed with a capillary DNA sequencer, ABI 310 (The Perkin–Elmer Co.). All mRNA contents were normalized with reference to G3PDH mRNA.

2.6. Statistical analysis

Multiple comparison was made by ANOVA followed by Scheffe's test. Otherwise, Student's t-test was applied.

3. Results

3.1. Estrogen-dependent cell proliferation of GH3

The relative cell numbers were measured at day 5 of treatment with E2 at concentrations from 10^{-13} to 10^{-9} M (Fig. 1). Significant stimulation of cell proliferation was observed at 10^{-12} M and the response appeared to reach a maximum at 10^{-11} M. The sizes of individual cells treated with E2 appeared to be larger than without hormone.

3.2. Estrogen-responsive genes identified by cDNA microarray

Differentially expressed genes based on the ratio of the measured hybridization intensities on GeneChip Rat Genome U34A between control and E2-treated cells are listed in Table 1. A minimal change of two-fold was applied to select up- and down-regulated genes. Two independent experiments were carried out and the genes showing reliable hybridization for both experiments were counted. The genes are listed according to average values of E2 induction. The results of ICI182780 treatment alone or with E2 are also given in Table 2. The genes regulated by E2 but not showing inhibition by ICI182780, which only accounted for four in total, are not included in the table. Interestingly, only 26 genes were categorized as up-regulated and seven as the down-regulated, out of approximately 8000 genes on the chip.

3.3. Confirmation of mRNA changes

From Table 2, the top ten genes and four others (#14, #15, #21 and #22) were selected and subjected to quantification of mRNA levels to confirm the results of cDNA microarray analysis. cDNAs from GH3 cells treated with E2 at 10^{-12} to 10^{-9} M and/or ICI at 10^{-7} M are examined and

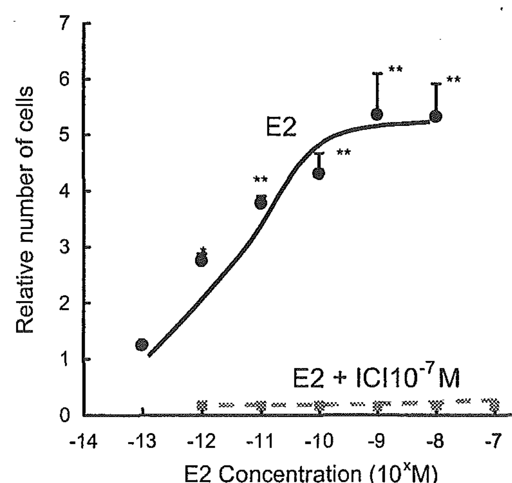

Fig. 1. Effects of 17β-estradiol (E2) and ICI182780 (ICI) on GH3 cell proliferation. Cells were seeded in 24-well plates at 1×10^4 cells per well. After five days treatment with E2 at 10^{-13} to 10^{-9} M alone or with ICI at 10^{-7} M, cell proliferation was measured by a modified MTT assay. Each point represents a mean ± S.E.M. ($n = 4$). *,** Indicates significant differences from the control value at 0.05 and 0.01, respectively.

Table 2
Genes up- and down-regulated by estrogen two or more fold in the microarray study

Genbank accession#		Gene name/blast match	Fold change in expression			
			E2(Expl)	E2(Exp2)	E2+ICI	ICI
Genes up-regulated						
#1	K00994	Calbindin-D9k*	8.12	6.20	0.70	0.33
#2	AI175539	Parvalbumin*	7.58	4.54	0.81	0.28
#3	AI014135	Ribosomal RNA*	6.23	4.93	1.17	0.94
#4	M17083	Alpha globin*	5.23	4.99	0.39	0.59
#5	S81478	3CH134/CL1 ATPase	4.77	4.12	0.97	1.11
#6	D26393	Type II hexokinase	2.75	3.15	0.14	0.49
#7	AI230712	PACE4*	2.98	2.73	0.44	0.15
#8	AF081366	K + channel ROMK2.1 isoform	3.21	2.44	0.88	0.20
#9	Y00396	c-*myc* protein	2.99	2.59	0.76	0.35
#10	U02553	Protein tyrosine phosphatase	3.32	2.23	0.67	0.44
#11	AF036548	RGC-32	3.47	2.05	1.12	0.37
#12	U53505	Type II iodothyronine deiodinase	2.26	2.87	0.77	0.34
#13	Y09507	Hypoxia-inducible factor 1	2.60	2.38	1.13	0.69
#14	U24175	Regulator of transcription 5a1	2.77	2.01	0.61	0.46
#15	D13623	p34 protein	2.43	2.32	1.05	1.02
#16	M58040	Transferrin receptor	2.37	2.38	0.73	0.30
#17	AA819776	EST (similar to HSP86)	1.93	2.76	1.82	1.97
#18	AA875126	EST (unknown)	2.33	2.27	0.58	0.70
#19	M14656	Osteopontin	1.89	2.69	1.37	1.22
#20	X67788	Ezrin, p81	2.28	2.23	0.47	0.50
#21	AA892522	EST (unknown)	2.19	2.23	0.60	0.82
#22	L16922	Progesterone receptor	2.30	2.04	0.89	0.67
#23	U57097	APEG-1 protein	2.36	1.97	1.43	1.51
#24	M24852	Neuron-specific protein	1.87	2.45	1.73	1.57
#25	AA817846	EST (similar to D-β-hydroxy butyrate dehydrogenase)	1.86	2.37	0.97	0.96
#26	AI169417	Phosphoglycerate mutase type B subunit mRNA*	1.98	2.23	0.97	0.92
Genes down-regulated						
	U67080	Zinc finger protein r-MyT3	0.49	0.47	1.38	1.06
	AA799964	EST (unknown)	0.49	0.41	0.51	0.71
	AI639263	EST (unknown)	0.46	0.41	0.68	0.32
	M27925	Synapsin 2a	0.47	0.35	1.31	1.31
	E03229	JP 1991272688-A/2	0.47	0.30	1.31	0.95
	AI237654	Vdup1*	0.40	0.35	0.81	0.84
	AA893280	EST (similar to adipose differenti-ation-related protein)	0.47	0.21	0.91	0.98

Gene are listed in order of average E2 fold change in Experiments 1 and 2. **Four E2 up-regulated genes were not inhibited by ICI, which are not included in this table (The GenBank accession numbers of these are AI138070, AA866485, D84480 and X74293).
* Indicates genes originally listed as ESTs but found to have perfect match by BLAST.

the results were summarized in Fig. 2. Although the fold increases of E2 induced gene expression were slightly lower than in the microarray analysis, up-regulation and inhibition by ICI182780 were confirmed except with three genes, #3, #6 and #15, which showed no responses. Time dependence of gene expression induced by E2 was also examined and the results are summarized in Fig. 3. As expected, some of the genes were expressed early after E2 administration and others increased gradually. Since the microarray analysis was carried out at only one time point, 24 h after E2 treatment, early responding and quickly muting genes would not be expected to be identified.

To determine E2 in inducing the transcription of genes #1 and #2, GH3 cells were treated with E2 in the presence of 0.5 μg/ml of actinomycin D (a transcription inhibitor) and 10 μg/ml cycloheximide (a translation inhibitor) for 3 and 24 h (Table 3). Increase in mRNA levels by E2 was blocked

Table 3
Effects of cycloheximide and actinomycin D on E2-induced mRNA change of calbindin D9k and parvalbumin in GH3 cells

	3 h	24 h
Gene#1: calbindin D9k		
Control	5.45 ± 0.70**	4.02 ± 0.33**
CHX	4.03 ± 0.11**	3.74 ± 0.27**
ActD	1.01 ± 0.21	1.13 ± 0.23
Gene#2: parvalbumin		
Control	1.81 ± 0.41	4.52 ± 0.94*
CHX	2.51 ± 0.19**	8.34 ± 0.37**
ActD	0.93 ± 0.09	1.58 ± 0.31

Cell were treated with E2 at 10^{-9} M for 3 and 24 h with or without cycloheximide (CHX) at 10 μg/ml or actinomycin D (ActD) at 0.5 μg/ml. The inductions by E2 were calculated for each treatment (mean ± S.E.M., $n = 4$).
* Indicates significant induction at 0.05 and 0.01, respectively.
** Indicates significant induction at 0.05 and 0.01, respectively.

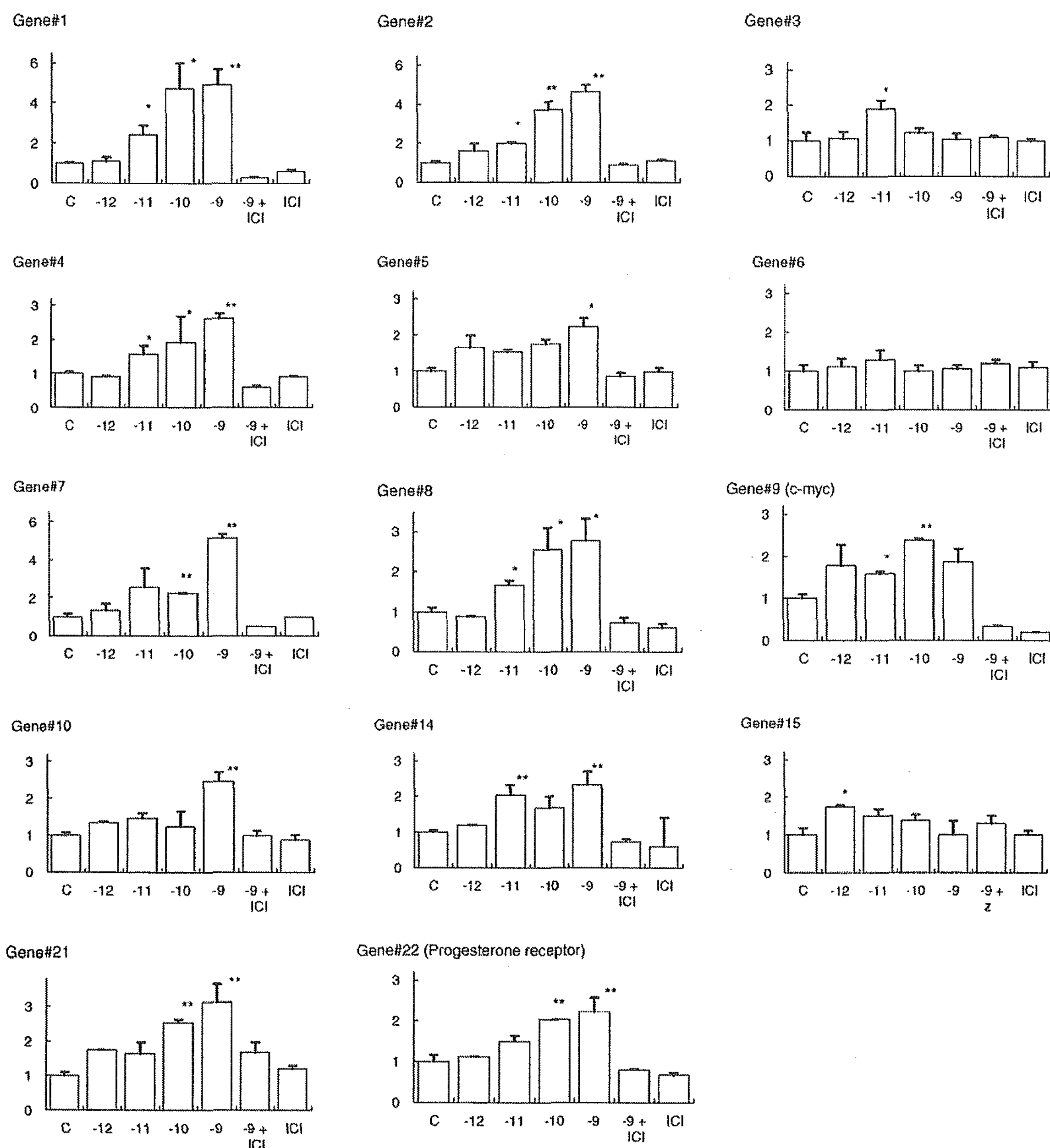

Fig. 2. Dose-dependent changes in gene expression levels measured by quantitative real-time RT-PCR. Cells were treated with different concentrations of E2 at 10^{-12} to 10^{-9} M and/or a single dose of ICI 182780 (ICI) at 10^{-7} M for 24 h. All mRNA contents were normalized with reference to G3PDH mRNA. The fold changes were calculated based on the gene expression in the cells treated with vehicle. Each point is an average of two independent experiments.

by actinomycin D but not by cycloheximide, which indicates that E2 regulates these genes at the transcriptional level.

3.4. Expression of genes in the pituitary gland

Expression of estrogen regulated genes in GH3 cells was further investigated in the anterior pituitary gland. First, mRNA expression of eleven-responsive genes was examined in short-term (24 h) and long-term (30 days) E2-treated ovariectomized F344 rats. Findings for estrogen-dependent increase for each gene are summarized in Table 4 as fold change of mRNA in E2-treated animals over that in the ovariectomized controls. All the genes except #4 were up-regulated in pituitary tissue by the short-term and long-term treatment of E2. Estrogen dependence of expression of gene #1 (calbindin-D9k) and gene #2 (parvalbumin) was extremely strong, over 100-fold induction being noted. For these and gene #9 (c-*myc*), more detailed time-dependent analysis was carried out. In Fig. 4, each mRNA level was calculated based on the level in ovariectomized rats at day

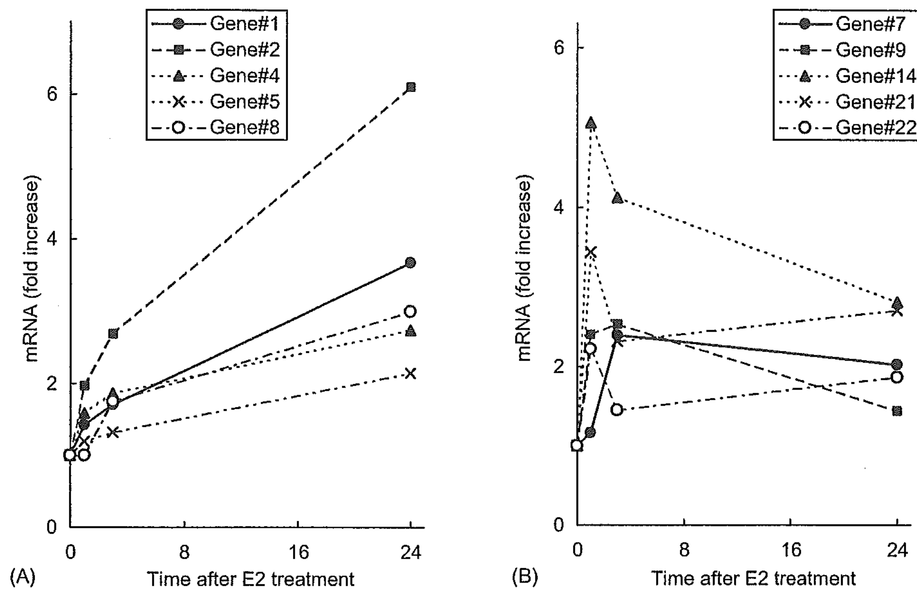

Fig. 3. Time-dependent change in gene expression levels measured by quantitative real-time RT-PCR. All mRNA contents were normalized with reference to G3PDH mRNA. Cells were treated with E2 at 10^{-9} M for 0, 1, 3 and 24 h. Each point represents a mean ± S.E.M. ($n = 4$). *,** Indicates significant differences from the control values at 0.05 and 0.01, respectively.

Table 4
Estrogen-responsive genes identified by the microarray study in the pituitary tissues in ovariectomized F344 rats

Gene	GenBank accession#	Fold change in expression	
		24 h	1 month
#1	K00994	118	95.0
#2	AI175539	28.9	70.0
#4	M17083	1.1	0.6
#5	S81478	2.3	2.0
#7	AI230712	2.9	4.7
#8	AF081366	9.9	2.0
#9	Y00396	4.5	17.7
#10	U02553	3.1	1.6
#14	U24175	2.4	4.0
#21	AA892522	2.0	5.1
#22	L16922	4.2	9.4

Ovariectomized F344 rats were treated subcutaneously with pellets containing E2 for 1 and 30 days. The gene expression was measured by quantitative real-time RT-PCR in pituitary tissue and the fold changes were calculated based on the mRNA level in ovariectomized controls at time 0 ($n = 5$).

0. All the three mRNAs, for calbindin-D9k, parvalbumin and c-*myc*, were induced significantly within 3 h of subcutaneous E2 administration, although the increase was most prominent for calbindin-D9k, with a 72-fold elevation. Higher levels were still maintained after a month of chronic E2 treatment.

4. Discussion

The GH3 cell line has been widely used to investigate the functions of somatolactotrophic cells, since regulation of its GH and prolactin production appears to be physiologically relevant with dependence on thyroid hormones, estrogen and glucocorticoid [11,12,19]. In the present study, we applied microarray analysis and identified a number of estrogen-responsive genes.

In terms of GH3 estrogen-responsiveness, there are two distinct parameters, prolactin synthesis and cell proliferation. However, reported sensitivity to estrogen has varied in the literature [4,13–15,20]. The inter-laboratory variation may be due partly to differences in strain, since GH3 has a rather old origin and has been widely used. Technical problems with charcoal treatment of serum for removing estrogenic substances may have had an impact in some cases [21]. The estrogenic activity of phenol red or related contaminants in common culture media was not recognized until Katzenellenbogen's group provided a convincing evidence [22]. Prior to the present microarray analysis, GH3 cells were examined in our culture conditions and found to be very sensitive to estrogen, exhibiting induction of cell proliferation in response to E2 at a concentration as low as 10^{-12} M. The high sensitivity on cell proliferation appears typical for pituitary cell lines, like the MtT/E-2 cell line we have established and another lactotrophic cell line, PR1 [4,23]. ERα is the major type of ER expressed in GH3 cells with a ratio to ERβ of 380:1 according to quantitative PCR (data not shown).

Recently, estrogen-responsive genes have been investigated by cDNA microarray in human breast cancers and the normal uterus [24,25]. However, the pituitary gland has not been explored for estrogen-responsive genes by this approach, to our knowledge. In the present microarray analysis, a relatively small number of genes were found to be

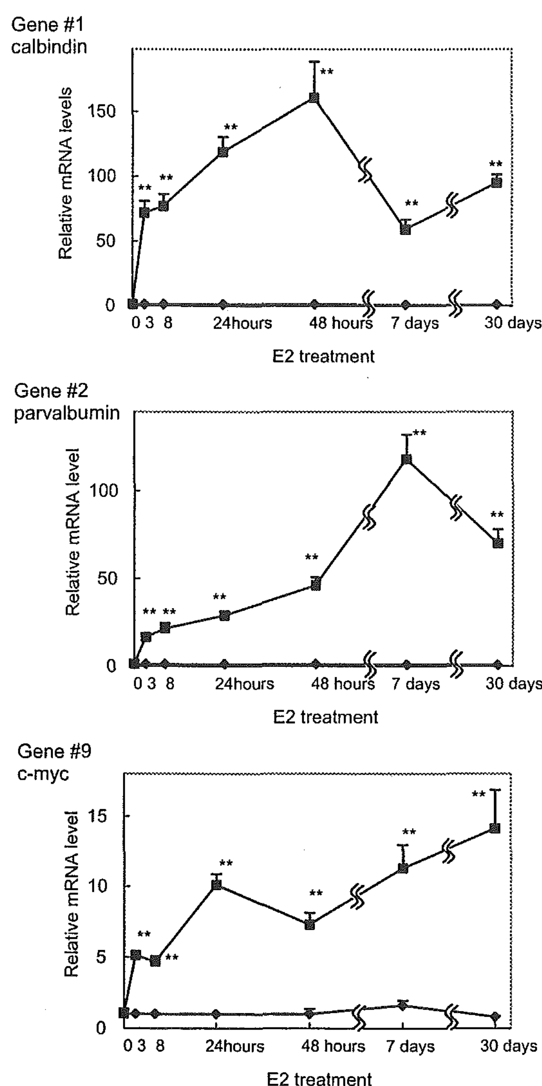

Fig. 4. Time-dependent analysis of three estrogen-responsive genes in the pituitary in vivo. Ovariectomized F344 rats were treated subcutaneously with pellets containing E2 for 3, 8, 24, and 48 h and 7 and 30 days. Gene expression was measured by quantitative real-time RT-PCR in pituitary tissue and fold changes were calculated based on the mRNA level in the ovariectomized controls at time zero. All mRNA contents were normalized with reference to G3PDH mRNA. Each point and bar represent mean ± S.E.M. ($n = 5$), *,** Indicates significant differences from the control values at 0.05 and 0.01, respectively.

regulated by estrogen with confirmation in most cases by quantitative real-time PCR. Suppression by ICI of E2-induced gene expression was also confirmed. The degrees of change were similar with real-time PCR analysis and GeneChip data and although we selected up-regulated genes after 24 h of estrogen exposure, some genes proved to be rapidly regulated (Fig. 3(B)) including these for the progesterone receptor and c-*myc*. Estrogen-responsive induction of progesterone receptor is well documented for the primary target, the uterus, as well as in the anterior pituitary gland [26,27]. Estrogen activation of c-*myc* also has been reported in the anterior pituitary gland and breast cancer cells [28,29]. A total of seven genes could be listed as down-regulated but they were not analyzed further, since all of them displayed relatively small degrees of change to 0.34–0.48 of the control values. Other known estrogen-responsive genes in the pituitary gland, such as prolactin and TGFα were not on the array used in the present study.

Interestingly, the in vivo expression of two genes, calbindin-D9k and parvalbumin, was found to be highly induced by E2 both in the short and longer term, which may suggest that hypothalamus or other indirect endocrine pathways would be involved in regulating genes in addition to the direct transcriptional activation. Calbindin-D9k is a vitamin D-dependent intestinal calcium-binding protein that is detectable in the duodenum, uterus and placenta [30–32]. Another vitamin D-dependent calcium-binding protein, calbindin-D28k, expressed in kidney and brain has no homology with calbindin-D9k either at the nucleotide or at the transcript levels [33]. The calbindin-D9k gene has been reported to contain a 15-base-pair imperfect palindrome with high homology to the estrogen- and glucocorticoid-responsive elements (ERE and GRE) [34]. Although there is no evidence that this protein is regulated by estrogen in the intestine through this motif, it is possible that the imperfect ERE is functional for the hormone-dependent transcription in the pituitary gland. Parvalbumin is another calcium-binding protein that belongs to the EF-hand calcium-binding protein like calbindin-D9k [35]. It is abundant in fast contracting/relaxing muscle fibers, where it plays a role as a calcium buffer and is also found in neurons as well as in endocrine glands including pituitary, thyroid, adrenals, testes and ovaries [36]. It has been postulated that parvalbumin can prevent cell death due to calcium overload in neurons. Although its expression is developmentally regulated in muscle, brain and other tissues, no evidence indicating hormonal regulation has been reported [37,38]. The 5′ flanking region of the gene seems to function as the promoter but it does not contain any motifs for estrogen-dependent transcription [39,40].

Since RNA was extracted from whole anterior pituitary tissue in the present study, it is not clear which types of cell actually contributed to the increase in mRNA levels. Chronic treatment of rats with E2 is known to result in the development of lactotrophic tumors [5]. The F344 strain is the most sensitive to E2 and somatolactotrophs of the pituitary become hyperplastic after exposure for a week and steadily proliferate thereafter. In the present study, major response of GH3 cells was cell proliferation so that some of the identified genes might be expected to be mitosis-related and involved in estrogen-induced pituitary hyperplasia/tumorigenesis. Although up-regulation of the calbindin-D9k and parvalbumin gene are evident on long-term treatment of E2, there was no obvious correlation with the time period for pituitary hyperplasia in contrast to the c-*myc* expression which steadily increase.

In conclusion, the present microarray analysis allowed identification of a number of estrogen-responsive genes in GH3 cells whose regulation appears biologically relevant in the pituitary gland in vivo. The actual significance of two calcium-binding proteins discovered to be prominently induced by E2 remains to be explored in the future.

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Inhibitory Effects of *Bifidobacterium longum* on Experimental Ulcerative Colitis Induced in Mice by Synthetic Dextran Sulfate Sodium

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Key Words
Ulcerative colitis · *Bifidobacterium longum* · Intestinal flora · Dextran sulfate sodium

Abstract
Background/Aims: The relationship between alterations in intestinal microflora and ulcerative colitis is still not clear. Whether improvement in bacterial populations might be a new strategy for prevention or treatment needs to be tested. ***Methods:*** Ulcerative colitis was induced in mice by oral administration of synthetic dextran sulfate sodium (molecular weight 54,000). Inhibitory effects of concomitant treatment with *Bifidobacterium longum* were assessed in terms of total colon length and severity of histological changes. In addition, changes of microflora and short-chain fatty acids were tested in fecal samples and compared before and after treatment. ***Results:*** Administration of *B. longum* significantly inhibited both shortening of total colon length and the severity of ulcerative colitis compared to controls. It was confirmed that the administered *B. longum* resided in the gut and blocked the decrease of lactobacilli in fecal samples in mice with dextran sulfate sodium-induced colitis. ***Conclusions:*** Oral administration of *B. longum* exerts marked inhibitory effects on ulcerative colitis in mice.

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Introduction

The etiology of ulcerative colitis remains unknown. In particular, while the relationship with the alteration of intestinal microflora is known to be important, the reasons are not clear. *Bifidobacterium*, Bacteroidaceae, Peptococcaceae, *Eubacterium* and *Lactobacillus* are in fact significantly reduced in the active phase of ulcerative colitis patients, in particular *Bifidobacterium longum*, *Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, *Eubacterium aerofaciens*, *Lactobacillus acidophilus*, and *Lactobacillus reuteri* [1].

Prophylactic and therapeutic effects of fermented milk have been discussed [2]. It has been demonstrated that the survival rate of rats given colon carcinogens and fed fermented milk is higher than that of the animals fed nonfermented milk [3]. Dietary lactobacilli have an important role in gastrointestinal ecology [4]. *B. longum*, lactic acid bacteria isolated from human feces, enhances the mucosal IgA response to dietary antigens and thus the host's immune system [5, 6]. Further, an inhibitory influence of *B. longum* on colon, mammary, and liver carcinogenesis induced by 2-amino-3-methylimidazo[4,5-f]quinoline, a food mutagen, has also been reported [7].

In the present study, effects of *B. longum* on synthetic dextran sulfate sodium (DSS)-induced ulcerative colitis

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[8, 9] were therefore investigated in mice. Further, since anaerobic bacteria, including *B. longum*, produce various kinds of organic acids, they might have influencing effects on colitis condition. Short-chain fatty acids (SCFAs) and lactate in the fecal samples as well as intestinal microflora were tested and compared before and after treatment.

Materials and Methods

Mice

Specific pathogen-free CBA/J(H-2k) female mice (Charles River, Kanagawa, Japan) aged 8 weeks were housed in our animal laboratory center. They were maintained under standard laboratory conditions and had free access to animal chow (CE-2, Nippon Clear, Tokyo, Japan) and drinking water throughout.

Administration of DSS and B. longum

B. longum OLL6001, isolated from human feces, were prepared as described previously [7]. In brief, the bacteria were inoculated into an EG medium, incubated anaerobically at 37°C overnight, and then harvested by centrifugation and resuspended in TPY medium.

Mice were divided into four groups in experiment 1: group A (n = 10) given 0.2 ml of TPY medium once a day for 7 days by oral gavage, group B (n = 10) receiving viable (6×10^{10} cfu) *B. longum* 6001 once a day for 7 days, group C (n = 12) given 3% (wt/vol) synthetic DSS (molecular weight 54,000; Meitoh Sangyo, Tokyo, Japan) and 0.2 ml of TPY medium once a day for 7 days, and group D (n = 12) receiving 3% DSS and viable (6×10^{10} cfu) *B. longum* 6001 once a day for 7 days. To see whether the results in experiment 1 are repeatable and effects of *B. longum* continue for 10 days, in experiment 2, mice were maintained for 10 days and were treated as in experiment 1: group A included 9 mice, group B 9 mice, group C 12 mice, and group D 10 mice.

Bacteriological Analysis

To avoid selection bias, 5 mice were selected at random in each group for bacteriological analysis before treatment. After treatment, the same mice as before were used for bacteriological analysis. A quantitative bacteriological analysis of fecal samples of all groups (n = 5) before administration of the test substances (DSS, TPY medium or *B. longum* 6001 cells) was performed according to the methods of Mitsuoka et al. [10–12]. Fecal samples of groups A and B (n = 5) and the entire large-intestine contents of groups C and D (n = 5) were also used for analyses after administration of the test substances. After 1 g of sample was suspended in 9 ml of anaerobic diluent [11, 12], serial 10-fold dilutions from 10^{-1} to 10^{-8} were prepared. Aliquots (0.1 ml each) were spread on 13 selective agar media (peptone novobiocin colimycin, modified LBS, BS, neomycin-Nagler, ES, PS, PO, RCN, PEES, TATAC, desoxycholate-hydrogen sulfide-lactose, modified neomycin sulfate-brilliant green-taurocholate-blood and modified *Bifidobacterium* agars) and 4 nonselective agar media (glucose blood liver with and without 5% defibrinated horse blood, tripticase soy with 5% defibrinated horse blood, EG and M10 agars) [10–12]. Plates inoculated for the determination of obligate anaerobic bacterial counts were incubated under anaerobic conditions by the steel wool method at 37°C for 48 h. TATAC, PEES and TS were incubated for 48 h and DHL agar for 24 h at 37°C under aerobic conditions. After incubation, preliminary identification of bacterial groups was done with reference to colony and bacterial morphology, the gram reaction, and aerobic and anaerobic growth [10, 11]. The results were expressed as log10 of colony counts per gram wet weight of sample.

Assay of Organic Acids

Quantitative analyses of SCFAs and lactate in fecal samples of all groups A (n = 5), B (n = 5), C (n = 9) and D (n = 10), before administration of test substances, were performed according to the methods detailed previously [13, 14]. After administration of the test substances, fecal samples of groups A (n = 5) and B (n = 5) and the contents of the large intestine of groups C (n = 9) and D (n = 10) were similarly employed for the determination of SCFAs and lactate contents. The high-pressure liquid chromatography system (model L-6200; Hitachi, Tokyo, Japan) was used in combination with a UV-VIS detector (model L-4200; Hitachi), the column (Shodex Ionpak KC-811 plus Ionpak KC-810P guard column, Showa Denko, Tokyo, Japan) being run at 63°C with a flow rate for the mobile phase (0.02% $HClO_4$) of 1 ml/min. Protein in the culture was precipitated by adding 7.5 µl each of Carrez-1 solution (53.5 g of $ZnSO_4 \cdot 7\ H_2O$/100 ml of distilled water) and Carrez-2 solution {17.2 g of K4 [Fe(CN)6]·3 H_2O/100ml of distilled water} and then dilution with distilled water until the total volume was 300 µl. The supernatant was used for the assay. SCFAs were spectrophotometrically determined at 430 nm after postcolumn coupling with bromocresol purple (108 mg) and $Na_2HPO_4 \cdot 12\ H_2O$ (2.86 g) in 1,000 ml of distilled water with a flow rate of 1 ml/min.

Morphological Analysis

All analyses were done in a blind fashion according to the method described previously [8]. After samples of colonic wall adhering to filter paper were fixed in 10% formalin solution (pH 7.2) with no pinning, the medial longitudinal length of each colon was measured. Histological examinations were done with HE staining after medial longitudinal sections were processed for embedding in paraffin. Severity of ulcerative colitis in one section of each colon was histologically graded on a scale from 0 to 4 using the modified standard scoring system [15]: 0 = normal; 1 = focal inflammatory cell infiltration including polymorphonuclear leukocytes; 2 = inflammatory cell infiltration, gland dropout, and crypt abscess formation; 3 = mucosal ulceration or five or more foci of gland loss with inflammatory cell infiltration; 4 = mucosal ulceration more than 1 mm in length or two or more areas of mucosal ulceration. Total colitis scores were counted after having added up pathological scores for three parts: right-side colon (ascending colon and cecum), transverse colon and left-side colon (descending and sigmoid colon, and rectum).

Statistics

For the bacteriological results, statistical significance of differences was assessed with the Bonferroni/Dunn multiple comparison test and the t test. The data for SCFAs and lactate were also statistically analyzed with the Scheffé multiple comparison and t tests. Pathology scores and colon lengths were statistically analyzed with the Mann-Whitney U test. All analyses were performed using Stat View version 4.0 statistical software (Abacus Concepts, Berkley, Calif., USA).

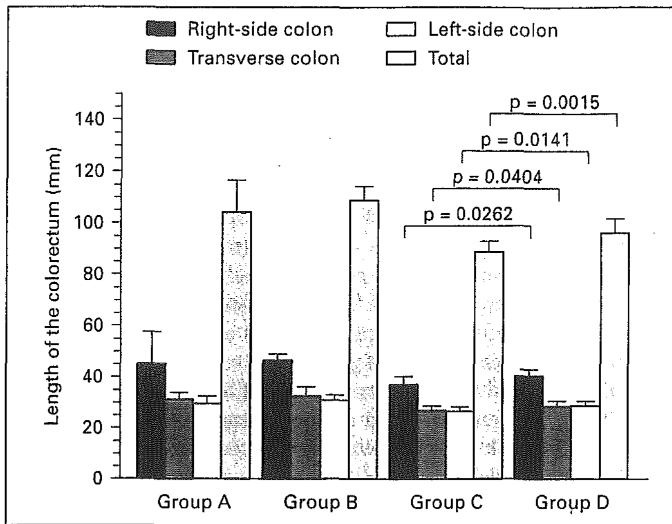

Fig. 1. Length of the colorectum in experiment 1 (day 7) in group A (drinking water + TPY medium), group B (drinking water + *B. longum*), group C (3% DSS + TPY medium), and group D (3% DSS + *B. longum*). Right-side colon = Length of the cecum and ascending colon; left-side colon = length of the descending colon, sigmoid colon and rectum; total = length of the colorectum and cecum.

Table 1. Pathology scores for ulcerative colitis (experiment 1, day 7)

	Group A	Group B	Group C	Group D
Right-side colon	0.4±0.5	0.3±0.5	2.0±0.4	1.6±0.5*
Transverse colon	0.6±0.5	0.4±0.5	3.3±0.6	2.7±0.5
Left-side colon	0.5±0.5	0.1±0.3	2.2±0.7	1.9±0.7
Total	1.5±0.7	0.8±0.6	7.4±1.1	6.4±1.0**

* p = 0.0455, ** p = 0.0404 vs. group C. Right-side colon = Cecum and ascending colon; left-side colon = descending colon, sigmoid colon and rectum

Results

Experiment 1 (Day 7)

Mean total colon lengths (mm) ± SD for groups A, B, C and D were 109.5 ± 4.0, 108.9 ± 5.5, 89.1 ± 3.7 and 96.1 ± 5.1, respectively (fig. 1), with a significant difference (p = 0.0015) between groups C and D. Differences in the lengths of each part [right-side colon (cecum and ascending colon), transverse colon and left-side colon (descending and sigmoid colon, and rectum)] were significant between groups C and D as follows: the length of the right-side colon: 36.9 ± 3.3 mm in C and 39.7 ± 3.0 mm in D (p = 0.0262); the transverse colon: 26.3 ± 2.0 mm in C and 27.9 ± 2.6 mm in D (p = 0.0404); the left-side colon: 26.3 ± 1.9 mm in C and 28.5 ± 2.0 mm in D (p = 0.0141).

The severity of induced colitis was greater in the transverse colon and left-side colon (descending and sigmoid colon, and rectum) than in the right-side colon (cecum and ascending colon). Mean total pathology scores (± SD) for groups A, B, C and D were 1.5 ± 0.7, 0.8 ± 0.6, 7.4 ± 1.1 and 6.4 ± 1.0, respectively (table 1), those for group D in the right-side colon and total colon being significantly lower than the group C values (p = 0.0455 and 0.0404, respectively).

Examination of the intestinal microflora in groups B and D after administration of the test revealed the presence of appreciable amounts of *B. longum* (8.24 ± 0.51, 7.53 ± 0.30) (table 2). Lactobacilli were significantly reduced ($p < 0.05$) in group C but not D after DSS administration compared to those before treatment. Staphylococci were significantly increased in group D after treatment.

Acetic acid in fecal samples or content of the large intestine was significantly increased ($p < 0.05$) as a result

Table 2. Influences of DSS and *B. longum* administration on microflora in feces [log(cfu/g)].

Organisms	Before treatment				After treatment			
	A	B	C	D	A	B	C	D
Total bacteria	9.48±0.13	9.53±0.07	9.55±0.12	9.43±0.23	9.75±0.28	9.59±0.12	9.13±0.13	9.60±0.26
Bifidobacteria	7.49±0.24 (5/5)	7.88±0.17 (5/5)	8.29±0.38(5/5)	7.57±0.19 (5/5)	8.11±0.35 (5/5)	8.52±0.23 (5/5)	7.53±0.21 (5/5)	7.96±0.24 (5/5)
B. longum	0 (0/5)	0 (0/5)	0 (0/5)	0 (0/5)	0 (5/5)	8.24±0.51 (5/5)	0 (0/5)	7.53±0.30 (4/5)
Bacteroidaceae	8.89±0.12 (5/5)	9.01±0.09 (5/5)	9.01±0.28 (5/5)	8.99±0.39 (5/5)	8.97±0.13 (5/5)	8.95±0.15 (5/5)	8.98±0.19 (5/5)	9.20±0.35 (5/5)
Lactobacilli	9.10±0.19 (5/5)	9.09±0.11 (5/5)	9.18±0.11 (5/5)	8.80±0.21 (5/5)	9.41±0.39 (5/5)	9.29±0.15 (5/5)	8.39±0.18 (5/5)*	8.76±0.20 (5/5)
Enterobacteriaceae	6.75±0.59 (5/5)	7.61±0.45 (5/5)	6.75±0.38 (5/5)	6.45±0.35 (5/5)	6.27±0.14 (5/5)	6.55±0.34 (5/5)	5.82±0.16 (5/5)	5.48±0.58 (5/5)
Streptococci	7.05±0.55 (5/5)	7.15±0.60 (5/5)	6.92±0.70 (5/5)	6.82±0.54 (5/5)	7.38±0.15 (5/5)	6.82±0.35 (5/5)	6.68±0.60 (5/5)	5.75±0.60 (4/5)
Staphylococci	4.10±0.10 (2/5)	4.92±0.28 (5/5)	4.82±0.30 (3/5)	4.45±0.21 (5/5)	4.40±0.29 (5/5)	4.59±0.29 (5/5)	4.70±0.34 (4/5)	5.01±0.11 (5/5)*

Before treatment: fecal samples are used in all groups (n=5).
After treatment: fecal samples are used in groups A and B (n ≒ 5) and large-intestine contents in groups C and D (n = 5). * $p < 0.05$ vs. before treatment.

Table 3. Influence of DSS and *B. longum* cells on organic acid contents in feces (m*M*)

Organic acid	Before treatment				After treatment			
	A	B	C	D	A	B	C	D
Total acid	23.8±17.1	37.8±14.3	56.2±32.8	57.3±16.2	43.7±17.2	61.4±12.2*	52.8±21.1	51.3±16.1
Acetic acid	12.4±4.9	16.0±3.7	20.3±9.7	20.6±9.7	16.7±3.5	22.6±6.0	34.2±9.2*	34.5±12.5*
Lactic acid	2.4±5.4	8.4±9.2	9.8±12.8	6.3±8.7	4.0±5.9	2.8±6.3	3.8±3.6	1.1±2.3
Propionic acid	0.0±0.0	0.0±0.0	0.8±2.4	3.9±4.0	3.2±4.5	6.8±4.1*	4.3±4.0*	4.6±3.8
n-Butyric acid	7.4±12.6	6.1±3.5	10.0±9.0	18.5±7.6	7.7±1.4	9.4±5.6	5.6±3.6	6.3±5.6*
Isobutyric acid	0.0±0.0	0.0±0.0	4.5±7.7	4.8±5.4	6.4±6.3	8.4±5.1*	3.4±5.1	2.1±4.5
Succinic acid	1.6±3.6	7.4±6.8	10.1±8.9	1.7±4.3	4.3±6.0	8.6±8.3	1.8±3.3	2.6±4.2
Valeric acid	0.0±0.0	0.0±0.0	0.6±1.9	0.0±0.0	0.0±0.0	1.0±2.3	0.1±0.4	0.4±0.9
Isovaleric acid	0.0±0.0	0.0±0.0	0.0±0.0	0.3±0.8	0.0±0.0	0.0±0.0	0.1±0.3	0.0±0.0
n-Caproic acid	0.0±0.0	0.0±0.0	0.1±0.4	1.1±3.5	1.4±3.2	1.8±4.0	0.0±0.0	0.0±0.0

Before treatment: fecal samples are used in all groups: A (n = 5), B (n = 5), C (n = 9), D (n = 10); after treatment: fecal samples are used in groups A and B (n = 5) and large-intestine contents in groups C (n = 9) and D (n = 10).
* $p < 0.05$ vs. before treatment.

of DSS administration in groups C and D after treatment (table 3). Propionic acid was significantly ($p < 0.05$) increased in group C after treatment. n-Butyric acid was significantly ($p < 0.05$) decreased in group D after treatment.

Regarding the relationship between microflora and organic acid contents, regression coefficients were calculated between viable counts of each bacterial strain and each organic acid concentration in feces (table 2). Significantly positive correlations ($p < 0.05$) were found between the total organic acid content and the number of streptococci, and between the lactate content and the number of lactobacilli.

Experiment 2 (Day 10)

Mean total colon lengths (mm) ± SD for groups A, B, C and D were 107.1 ± 6.9 (length of colorectum excluded cecum 85.0 ± 5.1), 104.4 ± 4.7 (82.8 ± 3.7), 90.0 ± 6.4 (71.7 ± 3.6) and 95.4 ± 7.2 (75.0 ± 6.5), respectively. While the value for group D was greater than those for group C, the difference did not reach statistical significance. Differences in the length of each part were not significant between groups C and D (data not shown).

Mean total pathology scores (± SD) for groups A, B, C and D were 1.0 ± 0.7, 0.7 ± 0.9, 8.4 ± 1.2 and 6.9 ± 1.4, respectively (table 4), with *B. longum* providing significant protection ($p = 0.0192$). Similarly, the pathology score of left-side colon was significantly lower ($p = 0.0321$) in group D than in group C.

Table 4. Pathology scores for ulcerative colitis (experiment 2, day 10)

	Group A	Group B	Group C	Group D
Right-side colon	0.2±0.4	0.1±0.3	2.3±0.5	1.9±0.6
Transverse colon	0.4±0.5	0.3±0.5	2.6±0.5	2.3±0.5
Left-side colon	0.3±0.5	0.2±0.4	3.6±0.7	2.7±1.0*
Total	1.0±0.7	0.7±0.9	8.4±1.2	6.9±1.4**

* $p = 0.0321$, ** $p = 0.0192$ vs. group C. Right-side colon = Cecum and ascending colon; left-side colon = descending colon, sigmoid colon and rectum.

Discussion

Although the DSS-induced colitis is an acute injury model, which may be different in the pathogenesis of human ulcerative colitis, the features in the model used here are relatively similar to those seen in patients with ulcerative colitis in terms of both clinical and histopathological characteristics, including diarrhea, occult blood, melena, mucosal inflammatory cell infiltration, crypt abscess formation and mucosal erosion. It has been suggested that DSS induces inappropriate macrophage and lymphocyte functions, changes in the luminal bacteria, and toxic effects, resulting in ulcerative colitis [8, 16–19]. Thus, the DSS-induced colitis model is appropriate for the investigation of potential treatment approaches [20].

In fact, protective effects of anti-interleukin-1β antibodies, recombinant interleukin-1 receptor type 1 and bradykinin B2 receptor antagonist were tested in this DSS colitis model [21, 22].

According to our previous experimental studies [8], 3% DSS administration for 7 or 10 days is considered to be appropriate for the induction of acute colitis. Further, the dose of viable *B. longum* was also determined to be 6×10^{10} cfu for every day of administration, since mucosal IgA or antigen-specific IgA levels rose with oral administration of *B. longum* (8×10^{10} cells) in mice [9]. In the present study, *B. longum* administered in groups B and D was found to reside in the gut after inoculation in experiment 1 (table 2), a decrease of bifidobacteria being inhibited in group D. Inhibitory effects on ulcerative colitis development were evident in terms of both shortening of the colorectum and severity of colitis in experiment 1 (day 7) and severity of colitis in experiment 2 (day 10).

With respect to the alteration of microflora and organic acid contents in fecal samples or large-intestine contents after administration of *B. longum*, an inhibition of the decrease of lactobacilli protected against DSS-induced colitis in the present study. In particular, a decrease of lactobacilli in DSS-induced colitis (group C) was apparent in line with our former study [8], and this was blocked in group D by *B. longum* administration, although direct evidence was not available in the present study. Administered *B. longum* might have changed fecal organic acids, which could have stimulated lactobacilli in group D [23]. However, it is difficult to discuss the changes of fecal organic acids as regards DSS-induced colitis development, because the variances of each value were too large to see the reliable results in the present study. In a previous report, Ariake et al. [24] suggested that the increase of succinic acid might be caused by DSS administration, resulting in a colitis condition. However, it was different in our experiment. Therefore, establishing the real relation between the alteration of fecal organic acids and colitis development is still controversial.

Further, n-butyric acid was decreased after treatment in groups C and D with DSS-induced colitis, similar to the previous report [24]. Butyrate is the main energy source of colonic epithelial cells. Therefore, the decrease of butyrate, possibly due to low production, might alter the microenvironment of the colonic mucosa and contribute to the colonic ulceration. On the other hand, acetic acid was significantly increased in groups C and D, suggesting a vicious influence on the colonic mucosa. Indeed, it has been suggested that the inflammatory condition in ulcerative colitis may result from a metabolic defect in SCFA oxidation in the colon [25].

To sum up, the findings suggest that oral administration of *B. longum* protects against DSS-induced colitis in mice. Further studies of this phenomenon are clearly warranted.

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A statistical method for judging synergism: application to an endocrine disruptor animal experiment

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SUMMARY

This article proposes a statistical method for judging whether or not the combined action of chemicals is synergistic, being focused on the case in which two or more endocrine disruptors are made to act simultaneously. After defining synergism, the synergistic relation of two chemicals is formulated for a higher response than that expected under an exchangeable relation between them. Using this formulation as a basis, we then rationalize the triangular design for an animal experiment in which all dose settings are controlled within a triangle domain that prescribes the sum of doses of simultaneously applied chemicals less than a certain level. In addition, a statistical test is proposed for judging the synergism among chemicals used in animal experiments, i.e. the test evaluates the discrepancy between the observed mean response from simultaneous administration groups of chemicals and an estimated response under the null hypothesis of zero interaction based on data from single administration groups. Finally, test performance is examined using a simulation study and a case study—the rodent uterotrophic assay. The simulation study revealed that the test is not superior in power to the standard analysis of variance test based on a linear model with interaction term, yet robust in the sense that type I errors under variance heterogeneity were better controlled using Welch correction than the analysis of variance test. The application of the proposed statistical test to an animal experiment is considered acceptable based on results. Copyright © 2003 John Wiley & Sons, Ltd.

KEY WORDS: animal experiment; endocrine disruptor; design of experiment; statistical test; synergism

1. INTRODUCTION

To protect people from the harmful effects of chemicals, society has begun regulating environmental pollutants and toxicants at levels having negligible impact. In the past these regulation levels were determined based on the knowledge or toxicity data of a single administration of an individual chemical. Recently, however, synergic effects due to combining chemicals have become apparent and regulations are now considered to be based on the knowledge or data on their combined action. Accordingly, many studies have been carried out to clarify synergism of harmful effects by

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simultaneous administration of chemicals (see, for example, Reif, 1984; Hasegawa *et al.*, 1996). One particular application is that for the synergism of endocrine disruptors.

As one of the authors has been engaged in endocrine disruptor studies underway in Japan (Kanno *et al.*, 2001), it was necessary to determine how to obtain and analyze data from animal experiments concerned with synergism. Under this requirement, here we investigate data collection/analysis allowing evaluation of synergism, applying the devised method to an animal experimental study conducted in Japan.

Section 2 explains the issues elicited in the above-mentioned study, while Section 3 discusses the concept of synergism adopted in the analysis. Sections 4 and 5 subsequently describe the experiment design and statistical test used for analysis of the endocrine disruptor study, after which Sections 6 and 7 respectively present the results of the simulation study, which examines the performance of the proposed test, and a case study. Section 8 provides a conclusion and discussions.

2. ENDOCRINE DISRUPTOR ISSUE

Chemicals that induce a hormonal effect are referred to as hormonally active agents (HAAs)—see, for example, Committee report (1999), EDSTAC (2001) and Solicitation (2001). Endocrine disrupting chemicals (EDCs) are defined as HAAs that induce adverse effects. As most hormonal effects are well known to be mediated by hormone receptors, endocrine disruption can therefore be defined as a 'receptor-mediated adverse effect or toxicity'.

A question arises concerning what are the major differences between traditional toxicity and receptor-mediated toxicity, especially that occurring through nuclear receptors such as estrogen and androgen receptors, or through ligand-inducible transcription factors such as dioxin receptors. It must be realized that the effects are mediated by the 'signal', and that the 'toxicants' do not need to be at the site of the adverse effect. In addition, with regard to the 'redundancy' of the receptor system, such receptors bind a variety of chemicals having various structures. Naturally, then, the affinity is different among chemicals and usually much lower than that of intrinsic natural hormones such as estradiol (see, for example, Yamasaki *et al.*, 2002). However, binding does occur, and if the concentration of the ligand goes above a certain level, then it usually has the capability to transduce the signal just as natural hormones do.

Since the signal transduction system basically amplifies the signal, it is believed that this occurs at a lower dose range than that exhibited in traditional toxicity studies. Expansion of this aspect may indicate that a system exists in which there is no threshold in response. Another aspect of redundancy is that each particular chemical can change the conformation of the ligand-bound receptor molecule according to the shape of each ligand molecule. If true, this may lead to different signaling properties especially when considering interactions with DNA and/or co-factor molecules.

Ligand-bound receptor molecules need to bind to a specific DNA sequence and recruit co-factors and other transcription machinery molecules in order to induce actual biological effects. In this context, the combined effect of multiple chemicals can be slightly different from what we expect from the monitored effect due to a single chemical.

Moreover, because more than one signaling system is present in humans, and because many other nuclear receptors/transcription factors are redundant in such ways, there may be an interaction between different signal pathways which leads to possible synergism for certain biological endpoints. Therefore, the definition currently needed for the expected combined effect is that if two treatments produce the same endpoint, they can be exchanged by any ratio to produce the same magnitude of the

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effect. The definition of an unexpected combined effect is that the effect due to such a combination is much larger than the particular effect induced by each treatment alone.

3. DEFINITION OF SYNERGISM

There are numerous discussions on the definition of additivity, synergism, and antagonism (see, for example, Rothman, 1980; Saracct, 1980; Reif, 1984; Berenbaum, 1989; Kodell and Pounds, 1990; Machado and Robinson, 1994; Laska *et al.*, 1997; Gennings *et al.*, 1997; Roy and Estieve, 1998).

From the 1920s to the 1960s, pharmacologists attempted to classify mechanisms representing the mode of combined action of two chemicals, which is the case considered here. Such trials subsequently generated numerous technical terms such as 'independent joint action,' 'similar joint action,' 'synergistic action,' 'dissimilar joint action,' 'potentiation,' 'depotentiation', and 'augmenter.' Due to the complexity of the concepts and difficulties in actual verification, such mechanistic analyses had virtually ended until a simple definition was introduced (Sakuma, 1996).

It is illustrated in a pharmacology textbook (see, for example, Laurence and Bennett, 1980) as a chart representing a 'Mountain of Happiness', which is an isobolic expression of happiness given after drinking a certain amount of wine followed by coffee. On this chart, a combination of a certain amount of wine and coffee realizes the apex of the response, which cannot be expected by the single administration of wine or coffee, while an excessive administration ends to dullness or sleep. It implies that the pharmacologically useful endpoint is to determine the best combination of two treatments (wine and coffee) regardless of mechanistic considerations. Synergism can be used to express such a peak in an isobologram, which also indicates that too much wine and/or coffee reduces happiness.

In general, toxicologic events are also complex, multi-step phenomena that are not fully understood; hence, it is reasonable to surmise that mechanistic considerations are not established for predicting the combined adverse effect of two chemicals. The definition of synergism regarding hazard identification must therefore be based on a non-mechanistic approach analogous to the Mountain of Happiness, although we are obviously not interested in the best combination of two chemicals that produce the strongest adverse effect. Our interest under the above-mentioned situation concerns the low dosage range in which two chemicals show combined adverse effects at a higher magnitude than that expected when two chemicals are equal in a particular response, i.e. they are exchangeable by any ratio. This viewpoint leads the following formulation adopted here.

Let $f(d_A, d_B)$ be the response at the combined dosage (d_A, d_B) of two chemicals A and B, and D_A and D_B be such that $f(D_A, 0) = f(0, D_B)$ under the assumption that f is a continuously monotone increasing function of either coordinate. If chemicals A and B are exchangeable, then $f(d_A, d_B) = f(D_A, 0) = f(0, D_B)$ is expected for (d_A, d_B) on the line connecting $(D_A, 0)$ and $(0, D_B)$. Accordingly, we define the response of the two chemicals to be 'synergistic' if $f(d_A, d_B) > f(D_A, 0) = f(0, D_B)$ for $(d_A, d_B), (D_A, D_B)$ such that

$$\frac{d_A}{D_A} + \frac{d_B}{D_B} = 1 \tag{1}$$

The case where the equality $f(d_A, d_B) = f(D_A, 0) = f(0, D_B)$ holds, implies 'zero interaction'.

The combined action of two chemicals considered here is, within a certain dose range, the same as the simple similar action for quantal response discussed by Hewlett and Plackett (1959) (see also Piegorsch and Bailer (1997) for summarized explanation), but is slightly different in the sense that it is formulated through an isobolic relation. This formulation is meaningful for proposing a triangular

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design, for we need not worry about the combined action of simultaneous administration of chemicals in the dose which is the maximum in the groups with individual chemical administration, while the formulation by Hewlett and Placket was too strict to apply to toxicity evaluation.

4. DESIGN OF EXPERIMENT

From a statistical viewpoint, synergism is examined experimentally using one-sided hypothesis testing for the null hypothesis of zero interaction. Note that a linear model can, without loss of generality, be assumed to express the dose–response relationship under the above-mentioned situation, i.e. in the exchangeable case.

This is true because the dose dependency of the response to both chemicals can be linearized by a suitable scale adjustment and a certain transformation of response, i.e. by the use of a function such as a link function in a generalized linear model that makes the dose–response relationship linear so that the relation $f(d_A, d_B) = \beta_0 + \beta_A d_A + \beta_B d_B$ holds.

While the factorial design shown in Table 1 is most often used for statistically evaluating interaction, it is not appropriate in our case, for the linearization should be confined within a certain dose range. In a two dimensional (2D) plane having coordinates that respectively indicate the dose of each chemical, responses outside the line connecting the maximum dose of the two chemicals do not provide any information on the synergism, so that the dose settings outside this triangle domain are useless for evaluating synergism.

In fact, even when the response for Groups (10), (12), (13), (14), (15), and (16) in Table 1 is quite high, it cannot be used to evaluate synergism because corresponding zero interaction response to be compared with them cannot be estimated. Consequently, we propose to use the triangular design, which eliminates the above-mentioned groups as shown in Table 2, for an animal experiment under the condition that the number of doses given by the administration of individual chemical is the same between the two chemicals. The number of simultaneous administration groups, which is 3 in Table 2, may well be dependent on the purpose of the experiment, but this is not our principal concern here.

5. STATISTICAL METHOD

The one-sided statistical test for evaluating the discrepancy between the observed response and the response estimated under the null hypothesis of zero interaction is considered reasonable as the statistical method for data analysis.

Table 1. An example of factorial design with 4 dose levels of each chemical. Animals are randomly allocated to each of 16 groups. Groups (1) through (7) correspond to single administration groups, whereas Groups (8) through (16) represent simultaneous administration groups

		Dose of chemical A			
		d_{A1}	d_{A2}	d_{A3}	d_{A4}
	d_{B1}	(1)	(2)	(3)	(4)
Dose of	d_{B2}	(5)	(8)	(9)	(10)
chemical B	d_{B3}	(6)	(11)	(12)	(13)
	d_{B4}	(7)	(14)	(15)	(16)

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Table 2. An example of triangular design with 4 dose levels of each chemical. Animals are randomly allocated to each of 10 groups. Groups (1) through (7) correspond to single administration groups, whereas Groups (8) through (10) represent simultaneous administration groups

		Dose of chemical A			
		d_{A1}	d_{A2}	d_{A3}	d_{A4}
	d_{B1}	(1)	(2)	(3)	(4)
Dose of chemical B	d_{B2}	(5)	(8)	(9)	
	d_{B3}	(6)	(10)		
	d_{B4}	(7)			

With the endocrine disruptor issue in mind, we assume that the observed variable y_{ij} of jth individual of ith group is distributed as normal with mean μ_i and variance σ_i^2 and that the ys are independent. Let $\bar{y}$ be the observed mean response for simultaneous administration groups (Groups (8), (9), and (10) in the case of Table 2) of two chemicals and $\hat{y}$ be the estimated response corresponding to $\bar{y}$ using data for groups (Groups (1) through (7) in the case of Table 2) with the administration of individual chemicals under the assumption of zero interaction. Naturally, $\bar{y}$ and $\hat{y}$ are statistically independent.

We propose using the following test statistic:

$$T = \frac{\bar{y} - \hat{y}}{\sqrt{\mathrm{Var}(\bar{y}) + \mathrm{Var}(\hat{y})}} \tag{2}$$

where $\mathrm{Var}(\bar{y})$ and $\mathrm{Var}(\hat{y})$ are the estimated variances of $\bar{y}$ and $\hat{y}$, respectively.

If we assume that all σs are equal, the denominator of the statistic T should be pooled within variance, with the degrees of freedom ν being equal to 'the total number of observations – the number of groups' and the critical value with significance level α is the upper 100α percentage point, $t(\nu, \alpha)$, of a t-distribution with degrees of freedom ν. Else if we assume that σs are homogeneous within simultaneous administration groups or groups with individual chemical administration, but heterogeneous between two classes, the two terms in the denominator of T should be separately estimated as the within-class sum of squares divided by 'the total number of observations of the class—the number of the groups in the class'. In the latter case, the critical value is set at $t(\nu, \alpha)$ with the degrees of freedom ν adjusted by Welch correction (see Welch, 1938, or Satterthwaite, 1946).

In the real situation of toxicity experiments, the variances are likely to be heterogeneous and even the latter assumption may be violated. However, since the heterogeneity of variances cannot be exactly estimated, we propose to use the latter test (Proposed-W) as the statistical method for judging synergism, or the former test (Proposed-T) when the homogeneity of variance is confirmed, the performance of these tests being compared with a regression test in the next section.

Thus, the flow of the proposed method is as follows:

Step 0. Check the linearity of the dose–response relationship for the groups with individual chemical administration. If a non-linear dose–response relationship is observed, transformations that linearize the relation are applied.
Step 1. Fit a linear response plane, i.e.

$$y = \beta_0 + \beta_A d_A + \beta_B d_B \tag{3}$$

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