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Safety Evaluation of Hypoallergenic Wheat Flour by Using a DNA Microarray

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The effect of the consumption of hypoallergenic wheat flour (HWF) on the expression of a wide spectrum of genes was analyzed by using a DNA microarray. Gene expression profiles in the liver and intestines of rats fed on a diet composed mainly of HWF were compared with those of rats fed on a normal flour-based diet. Among the eight thousand transcripts represented on the GeneChip microarray, no more than 30 genes exhibited up- or down-regulation of two-fold or more after one week or two months of HWF consumption. No adverse effects were apparent. Up-regulation of some of the genes known to respond to the interferon-γ signal was apparent in the one-week experiment, which may be related to possible oral immunotolerance resulting from HWF feeding. This DNA microarray technology presents an efficient method for evaluating the safety of foods.

Key words: wheat flour; DNA microarray; allergenicity; food safety

Intensive attempts are being made to develop and utilize new food materials and new food components that effectively help to improve health and prevent diseases.[1)] In the course of such research and development, establishing the safety of these foodstuffs is of primary significance.[2)] In many cases, the analysis of food safety adopts approaches similar to those used for pharmaceuticals. Considering that the mode of action of food is totally different from that of medicines, novel indices to ascertain safety need to be utilized. Improving the efficiency of such safety analyses is a prerequisite for the facilitating food development. An analytical method for monitoring a wide variety of cellular responses would be particularly effective for this purpose.

DNA microarray technology, the use of which is continuously expanding, enables simultaneous analysis of the expression of thousands of genes.[3,4)] This method, which has already had a strong impact on many aspects of life sciences, is likely to be a valuable tool for food safety analysis.[5)] We attempt in the present study to apply a DNA microarray to analyze the safety and functionality of hypoallergenic wheat flour (HWF) developed by the Watanabe group.[6)] This HWF is produced by treating flour with cellulase and actinase and is widely used by patients with wheat allergies.[7)] It not only has high effectiveness for preventing wheat allergy, but may also induce oral immunotolerance.[8)] Moriyama *et al.* have shown with rats fed on this material that it has reasonable nutritional value and that months of its intake resulted in no symptoms of toxicity in terms of the body weight gain, serum indices, or other parameters.[9)] A broader analysis of the safety of this material as well as exploring its new functionality need to be addressed.

We fed rats with an HWF diet for short- and long-term periods and obtained gene expression profiles from their liver and intestines. The expression pattern of the HWF-fed rats was compared with that of control rats to discern whether the intake of HWF caused any adverse effects with respect to the gene expression.

Material and Methods

Animals and feeding experiments. Five-week-old male Wistar rats were purchased from Charles River Japan (Kanagawa, Japan). The rats were kept in a room maintained at $22 \pm 1\,^{\circ}C$ with a 12-h light:dark cycle

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Abbreviations: HWF, hypoallergenic wheat flour; NWF, normal wheat flour; IFN-γ, interferon-γ; IgE, immunoglobulin E

Table 1. Composition of the Diets

Ingredient	NWF	HWF	12% Casein
		(g/kg)	
Casein	70[d]	70[d]	140[d]
NWF[a]	750[e]	0	0
HWF[b]	0	750[e]	0
Cornstarch	28	28	656
Soybean oil	50	50	50
Mineral mixture[c]	40	40	40
Vitamin mixture[c]	10	10	10
Cellulose	50	50	100
Choline chloride	2	2	2
Methionine	0	0	2

[a]NWF, normal wheat flour
[b]HWF, hypoallergenic wheat flour
[c]The mineral and vitamin mixtures (obtained from Oriental Yeast Co., Tokyo, Japan) were prepared according to the American Institute of Nutrition.
[d]Protein content was 85%.
[e]Protein content was 6.5%.

(lights on at 8:00). After they had been given a 12% casein diet[10] between 10:00 and 18:00 for 3 days, the rats were given an experimental diet for a short (7-day) or long (59-day) period. The compositions of the normal wheat flour (NWF) and HWF diets are shown in Table 1. The NWF and HWF diets were composed of 75% NWF or HWF, 7% casein, and other nutrients. Both NWF and HWF were provided by Omu Milk Product Co., Ltd.

On the 8th day (short-term) or the 60th day (long-term), the rats were given their respective diets for 1.5 hr and then anesthetized with pentobarbital. The liver and small intestine were excised, quickly frozen in liquid nitrogen and stored at $-80\,^{\circ}C$ until needed for use. The protocol for the animal experiments was approved by the Animal Use Committee of the Faculty of Agriculture at The University of Tokyo.

DNA microarray analysis. Total RNA extracted from the liver and small intestine (jejunum) of six rats fed on each diet was pooled and subjected to a DNA microarray analysis by the GeneChip system (Rat Genome

Table 2. Genes Highly Up- or Down-Regulated in the Liver by Short-Term Feeding with a Hypoallergenic Wheat Flour Diet

Up-Regulated

Accession no.	Description	Fold Change
*Z18877	2′-5′ oligoadenylate synthetase	6.4
X52711	Mx1 protein	5.5
X52713	Mx3 protein	5.3
Y07704	BEST5	5.2
*X53588	glucokinase	4.1
X57523	mtp1 4	3.7
L23128	MHC class I	3.5
U30186	growth arrest and DNA-damage-inducible protein GADD153	3.1
*U75397	Krox-24	3.1
L29281	initiation factor-2 kinase (eIF-2a)	3.0
S62516	SA {alternatively spliced}	2.8
U66723	purine-selective sodium/nucleoside cotransporter (rCNT2)	2.7
*M18416	nerve growth factor-induced (NGFI-A)	2.7
*AF065438	mama	2.7
*M58634	IGF binding protein-1 (rIGFBP-1)	2.7
J03959	uricase	2.6
AJ222813	precursor interleukin 18 (IL-18)	2.5
*AF023087	nerve growth factor induced factor A	2.3
*D10729	proteasome subunit RC1	2.2
*D11445	gro	2.2
*X61381	interferon induced mRNA	2.2
*J02722	heme oxygenase	2.1
*U10894	mRNA expressed in carotid artery tissue	2.1
*U77777	interferon-gamma inducing factor isoform alpha precursor (IGIF)	2.1
*L21711	galectin-5	2.0

Down-Regulated

Accession no.	Description	Fold Change
AF012887	sip9	5.6
X59864	ASM15	4.4
A09811	BRL-3A binding protein/IGFBP-2	3.5
*AB000199	cca2	3.5
*M91595	insulin-like growth factor binding protein 2	2.6

*Genes whose basal expression was detected as "present."

Table 3. Genes Highly Up- or Down-Regulated in the Small Intestine by Short-Term Feeding of a Hypoallegenic Wheat Flour Diet

Up-Regulated

Accession no.	Description	Fold Change
S58528	integrin alpha v subunit	16.0
X52713	Mx3 protein	10.6
X56228	rhodanese	8.0
AF100470	ribosome attached membrane protein 4 (RAMP4)	6.1
M74439	UDP glucuronosyltransferase	5.7
S74907	protein phosphatase 1M 110-kda regulatory subunit	5.3
*AF029240	MHC class Ib	5.3
AJ006710	phosphatidylinositol 3-kinase	4.3
*Y07704	BEST5	4.3
S81289	IgM kappa chain variable region	4.0
*Z18877	2′-5′ oligoadenylate synthetase	3.5
Y00396	c-myc oncogene and flanking regions	3.2
M13100	long interspersed repetitive DNA sequence LINE3 (L1Rn)	3.0
S75991	voltage-dependent sodium channel alpha subunit	2.8
*U49930	ICE-like cysteine protease (Lice)	2.8
AF030087	activity and neurotransmitter-induced early gene 2 (ania-2)	2.8
*S63521	glucose-regulated protein GRP78	2.6
*AF065438	mama	2.6
U17260	arylamine N-acetyltransferase-1 (AT-1)	2.6
AF034896	olfactory receptor-like protein (SCR D-8)	2.5
*U76714	cell adhesion regulator (CAR1)	2.5
*M10094	MHC class I truncated cell surface antigen	2.5
S82233	rBSC2=Na-K-Cl cotransporter homolog	2.5
*J02722	heme oxygenase	2.3
*M31038	MHC class I non-RT1.A alpha-1-chain	2.3
D31838	wee1 tyrosine kinase	2.3
L00686	carcinoembryonic antigen-related protein	2.3
*M77362	Ig non-productively rearranged lambda-chain	2.3
*L07399	(hybridoma 56R-7) immunoglobulin rearranged gamma-chain	2.1
X52711	Mx1 protein	2.1
L08100	glycam 1	2.1
AF034900	olfactory receptor-like protein (SCR D-7)	2.1
*U42719	C4 complement protein	2.1
D29646	ADP-ribosyl cyclase	2.0
AF072124	caspase-7	2.0
U60977	RAREG-2.2	2.0
L07410	(hybridoma 57R-1) immunoglobulin rearranged kappa-chain	2.0
M85300	sodium-hydrogen exchange protein-isoform 3 (NHE-3)	2.0
*M15114	DNA polymerase alpha	2.0
D28560	phosphodiesterase I	2.0
M74152	prolactin receptor	2.0

Continued on the next page.

U34A array, Affymetrix, Santa Clara, CA, USA) as previously described.[10] The hybridization signals were analyzed by using GENECHIP analysis suite ver. 5.0.

Results

The body weight gain (g/day and total gain rate) and food intake (g/day) were no different between the two dietary groups (data not shown). Tables 2 through 5 list the genes whose expression levels differed between the two groups by twofold or more. The genes marked by an asterisk in the tables are those whose expression levels were identified as "Present" in both groups by the GeneChip algorithm (high-expression genes). In other words, the expression levels of those genes without an asterisk were judged "Absent" or "Marginal" in at least one of the groups (low-expression genes). In respect of the high-expression genes, 14 were up-regulated and two were down-regulated in the liver after one week consumption of the HWF diet (Table 2). These numbers were 25 and 5, respectively, when the low-expression genes are included. Even fewer genes were affected in the 2-month feeding experiment; only five were down-regulated and none exhibited twofold or more up-regulation (Table 4). Five and twelve genes were up- and down-regulated, respectively, when low-expression genes are included. These changes were not directly related to such typical adverse effects as oxidative stress, carcinogenesis or deformity, in that no concordant changes of a group of genes relating to such phenomena were apparent.

In the small intestine, 41 genes were increased and 22 genes were decreased by short-term feeding (Table 3). These numbers drop to 14 and 14 when low-expression

Table 3. *Continued.*

Down-Regulated

Accession no.	Description	Fold Change
D84487	PMSG-induced ovarian	7.0
*U88324	G protein beta1 subunit (rGb1)	4.6
*M27467	heart cytochrome oxidase subunit VIc (COX-VIc)	4.3
S65091	cyclic AMP-regulated phosphoprotein	3.5
*AB013454	NaPi-2 beta	3.0
*M89646	ribosomal protein S24	3.0
*X59677	M2 gene	2.6
K01934	spot 14	2.6
*L17127	proteasome RN3 subunit	2.6
*U75927	cytochrome oxidase subunit VIIa	2.5
J02585	stearyl-CoA desaturase	2.5
*D90038	70-kDa peroxisomal membrane protein(PMP70)	2.3
*AF007107	soluble cytochrome b5	2.3
*AJ007016	protein tyrosine phosphatase	2.3
*X73911	amiloride binding protein (long form)	2.3
U48220	cytochrome P450 2D18	2.1
*AF095741	MG87	2.0
D49785	protein kinase (MUK)	2.0
*X78855	cation transporter	2.0
*AB000098	MIPP65	2.0
U59809	mannose 6-phosphate/insulin-like growth factor II receptor	2.0
U56839	P2u receptor protein	2.0

*Genes whose basal expression was detected as "present."

Table 4. Genes Highly Up- or Down-Regulated in the Liver by Long-Term Feeding of a Hypoallegenic Wheat Flour Diet

Up-Regulated

Accession No.	Description	Fold Change
AB016160	GABAB receptor 1c	14.9
M31032	contiguous repeat polypeptides (CRP)	4.0
U26595	prostaglandin F2a receptor regulatory protein precursor	2.3
L15618	casein kinase II alpha subunit (CK2)	2.0
Y15054	70 kDa tumor specific antigen	2.0

Down-Regulated

Accession No.	Description	Fold Change
S68589	set=Set alpha isoform	4.6
*M11794	metallothionein-2 and metallothionein-1	2.5
*M22360	plasma proteinase inhibitor alpha-1-inhibitor III group 3	2.5
S79676	interleukin-1 beta-converting enzyme	2.5
S81448	type I 5 alpha-reductase	2.5
M33550	cytochrome P450 15-beta (CYP2C12)	2.3
U21662	beta-1,2-N-acetylglucosaminyltransferase II (Gnt II)	2.3
Z27118	heat shock protein 70	2.3
AJ000556	Janus protein tyrosine kinase 1, JAK1	2.1
*S66184	lysyl oxidase	2.1
*S76054	cytokeratin-8	2.1
*S63233	phosphoglycerate mutase type B subunit	2.0

*Genes whose basal expression were detected "present."

genes are excluded. Only a few genes exhibited marked change in the intestines of the rats fed on the experimental diet over the long term (Table 5). None of these changes was strongly linked to any apparent adverse effects.

In both the liver and the intestines, the genes up-regulated by short-term feeding include those related to the immune system (Table 6). Among them were 2′-5′ oligoadenylate synthetase, Mx1, Mx3, and BEST 5 which have been reported to be induced by the interferon-γ (INF-γ) signal.[11–13] No induction of these genes was apparent with long-term feeding.

Discussion

The increasing number of patients with food allergies,

Table 5. Genes Highly Up- or Down-Regulated in the Small Intestine by Long-Term Feeding of a Hypoallergenic Wheat Flour Diet

Up-Regulated

Accession No.	Description	Fold Change
*L20869	pancreatitis associated protein III (PAPIII0)	7.5
*M98049	pancreatitis-associated protein (pap)	6.5
V01225	amylase	6.1
M35601	alpha-fibrinogen	3.7
*U32681	ebnerin	2.6

Down-Regulated

Accession No.	Description	Fold Change
S66618	multidrug-resistance transporter P-glycoprotein	2.3
AF039832	homeobox protein (rPtx2)	2.3
*Z75029	hsp70.2 mRNA for heat shock protein 70	2.1
Z27118	heat shock protein 70	2.1
*X59859	decorin	2.0

*Genes whose basal expression was detected as "present."

Table 6. Immune System-Related Genes Up-Regulated by Short-Term Feeding of a Hypoallergenic Wheat Flour Diet

Liver

Function	Gene	Fold Change
Resistance to virus infection		
*Z18577	2′-5′ oligoadenylate synthetase	6.4
Interferon induced		
X52711	Mx1 protein	5.5
X52713	Mx3 protein	5.3
Y07704	BEST5	5.2
AJ222813	precursor interleukin 18 (IL-18)	2.5
*X61381	interferon induced mRNA	2.2
*U77777	interferon-gamma inducing factor isoform alpha precursor (IGIF)	2.1
Immuno response to antigen		
L23128	MHC class I	3.5
Chemokine family		
*D11145	gro	2.2

Small intestine

Function	Gene	Fold Change
Resistance to virus infection		
*Z18877	2′-5′ oligoadenylate synthetase	3.5
Interferon induced		
X52713	Mx3 protein	10.6
*Y07704	BEST5	4.3
X52711	Mx1 protein	2.1
Immuno response to antigen		
AF100470	ribosome attached membrane protein 4 (RAMP4)	6.1
*AF029240	MHC class Ib	5.3
S81289	IgM kappa chain variable region	4.0
*M10094	MHC class I truncated cell surface antigen	2.5
*M31038	MHC class I non-RT1.A alpha-1-chain	2.3
*M77362	Ig non-productively rearranged lambda-chain	2.3
*L07399	(hybridoma 56R-7) immunoglobulin rearranged gamma-chain	2.1
L07410	(hybridoma 57R-1) immunoglobulin rearranged kappa-chain	2.0
Response to allergy		
U17260	arylamine N-acetyltransferase-1 (AT-1)	2.6

*Genes whose basal expression was detected as "present."

which can inflict unbearable pain on the sufferer, underscores the importance of developing effective treatments for food allergies. The wheat-associated allergy tends to exhibit very severe symptoms and has even been associated with life-threatening anaphylactic shock.[14] As wheat is a staple food in many countries,

eliminating wheat from a patient's diet is extremely difficult.[15] In this regard, attempts have been made to manufacture and utilize effective alternatives to wheat flour. Enzymatic modification of wheat flour to reduce its allergenicity is one of the practical approaches to this problem. It has been reported that a major allergen in wheat flour is the low molecular-weight glutenin in gluten that has an IgE-binding epitope.[16–18] Watanabe *et al.* have developed a method for producing HWF by decomposing gluten enzymatically.[6] They observed that it had low allergenicity and was highly effective for allergic patients.[7] An investigation on its chemical and nutritional properties made by Moriyama *et al.* with rats has shown that it had a nutritional value comparable to that of a simulated amino acid diet.[9] This study also addressed the safety of consuming HWF and showed that it caused no symptoms of toxicity in terms of growth parameters, organ weight and biochemical indices.

We focused in the present study on the effects of an HWF intake on the gene expression profiles in the liver and intestines. The DNA microarray used in this study contained about 8000 different rat transcripts. Among them, 2000–4000 genes were judged as "present" (expression detected) in each tissue. Although an extremely high amount of HWF (comprising 75% of the diet) was consumed by the rats, only a limited number of genes exhibited up- or down-regulation in response to HWF feeding. Compared with the case of the effect of the dietary protein source being evaluated,[10] for instance, the number of altered genes in the HWF group was remarkably low. As far as we know, there were no adverse effects as judged by the functions of the high-response genes (Table 2 to 5). For example, no groups of genes that are known to be directly involved in carcinogenesis or oxidative stress were affected much by HWF feeding. We therefore conclude that the consumption of HWF had an almost negligible impact on the general functions of the body.

The only exception was the mild up-regulation of INF-γ-responsive genes seen from the short-term feeding of HWF. The role of an increased mRNA level is not yet known, but we suspect that this phenomenon may be related to an alteration of the immunoresponses of HWF-fed animals. In fact, Watanabe *et al.* have shown that the consumption of HWF was likely to cause a state of oral immunotolerance by using an airway inflammatory rat model.[8] In the short-term (7-day) HWF-feeding experiment, the up-regulated genes in the liver and intestines included those related to the immune system, most of which were IFN-γ-responsive genes. IFN-γ is secreted from Th1 cells and has a function that suppresses the production of IgE.[19] Previous results have demonstrated that the serum IgE level in HWF-fed rats was equivalent to that of an NWF-fed group, although it was significantly lower than that of amino acid mixture diet group.[8] In addition, no increase in the gene expression of IFN-γ itself was detected in our experiments (data not shown). Although the consequence of the up-regulation of the IFN-γ-responsive genes is still unclear, there is the possibility that a topical response through an IFN-γ-related signaling pathway may be one of the causes for immunotolerance. The results do not necessarily mean that the product of these genes would directly affect the immunoresponse; other genes downstream of the IFN-γ signal may play more important roles. The status of IFN-γ signaling, including the phosphorylation of STAT1,[20] being actually up-regulated by HWF feeding needs to be addressed. Also important are an analysis of the Th1/Th2 balance and identification of the molecules that directly modulate IgE production in this situation.

Compared with the short-term experiment, fewer genes were altered in the long-term feeding experiment, in which no up-regulation of IFN-γ-related genes was apparent. Some sort of adaptive response may have been involved in the temporal alteration of gene expression.

Another interesting change specific to short-term feeding was the tendency for alteration of the genes relating to growth factors and their signaling such as the components of the insulin-like growth factor system and nerve growth factor signaling. Some of the changes facilitate and others inhibit the signaling pathways. Although the overall consequence of these changes is not known, one possibility is the role in the local and minute regulation of cell growth or cell survival which may help in maintaining normal functioning of the organs.

The gene expression profile in the intestines was analyzed in the present study since it is the site for digestion and absorption of ingested food and is also an organ carrying a specific immune system, the gastrointestinal immune system. An analysis of the gene expression profile in other organs, especially the organs for general immunity such as the spleen, would be useful in gaining more insight into the mechanism of possible immunotolerance.

DNA microarray technique has been applied to almost all aspects of life science and shown to be a highly effective tool for the function mining of food. The results of the present study indicate that this technique would also be applicable to the field of food safety analysis. The DNA microarray-based method is expected to promote food safety evaluation technology. The establishment of a standardized method for this purpose is a prerequisite for bringing it into broader practical application. This study may provide the primary information for achieving this goal.

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