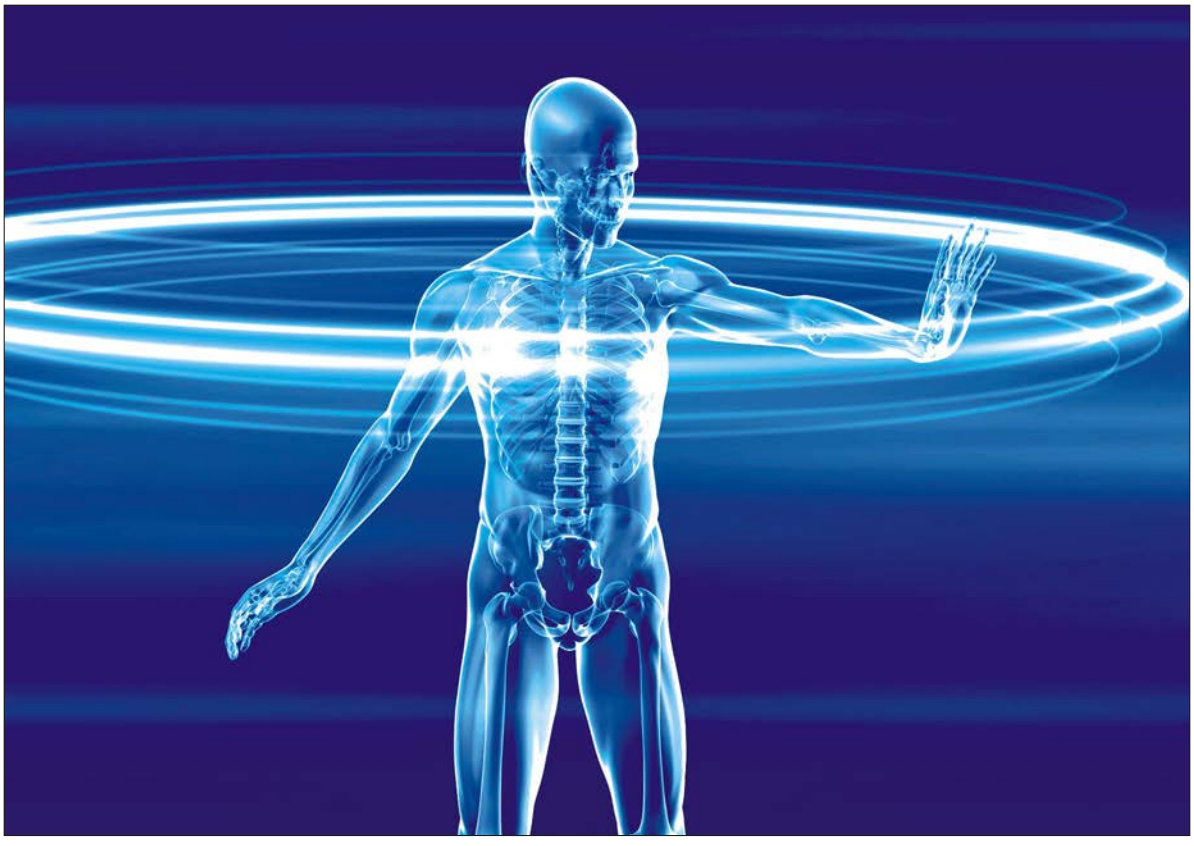

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Differences in Gene Expression Profiles among the Proximal, Middle and Distal Peyer's Patches in the Mouse Small Intestine

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ABSTRACT

Approximately 6-11 Peyer's patches are present in the mouse small intestine. However, to the best of our knowledge, few studies have reported the regional differences in mouse Peyer's patches. This study aims to determine whether regional differences exist in the immunological activation status and immunological functions of mouse Peyer's patches in the normal state. The most proximal Peyer's patches, the most distal Peyer's patches and the Peyer's patches nearest to the midpoint between the pylorus and ileocecal junction were obtained from the mouse small intestine. The gene expression levels in the PPs obtained from different regions were compared using the DNA microarray technique. Of the 187 genes that were expressed differently among the Peyer's patches from different regions, 6 genes were related to immune system process. The 6 genes that significantly differed in their expression levels were histocompatibility 2, q region locus 1 (*H2-Q1*); interleukin-18 (*Il18*); adenosine disaminase (*Ada*); angiogenin, ribonuclease A family, member 4 (*Ang4*); chemokine (c-c motif) ligand 6 (*Ccl6*) and histocompatibility 2, T region locus 22 (*H2-T22*). These findings suggest that the regional differences among Peyer's patches in mice in terms of the immunological activation status and immunological functions may be subtle.

Key words: Peyer's patch, microarray, regional differences, small intestine, mouse

INTRODUCTION

Peyer's Patches (PPs) are macroscopically visible lymphoid aggregates located opposite the mesenteric attachment of the small intestine. In some animal species like ruminants, 2 types of PPs have been reported: jejunal PPs and ileal PPs (Yasuda *et al*., 2006). The formers act as secondary lymphoid organs and the latter as primary lymphoid organs. To the best of our knowledge, however, few studies have reported the regional differences among PPs of the mouse small intestine, although approximately 6–11 PPs usually exist from the pylorus to the ileocecal junction in mice (Prinz *et al*., 2003).

The small intestine exhibits regional differences in terms of enzyme activity (Ware and Svensson, 1996), absorption (Gerard *et al*., 1996), intestinal flora (Smith, 1965), histological features (Hummel *et al*., 1975), etc. In a previous study, we identified regional variations in the intraepithelial lymphocytes of the mouse intestine (Suzuki *et al*., 2002; Suzuki and Yamamoto, 2006; Suzuki, 2009).

DNA microarray technique is one of the latest advances in the field of molecular biology and medicine. This technique has been widely used to measure expression levels of genes in microbes (Maqbool *et al*., 2006) and plants (Xoconostle-Cazares *et al*., 2010), as well as animals and human, in the research of physiology (Endo *et al*., 2011), toxicology (Gato and Means, 2011), oncology (Osareh and Shadgar, 2009), immunology (Lim *et al*., 2009) and etc.

The purpose of this study was to determine whether the proximal, middle and distal PPs of the mouse small intestine exhibit regional differences in terms of the immunological activation status and immunological functions in the normal state by means of comprehensive gene expression analysis using DNA microarray technique.

MATERIALS AND METHODS

Animals: Four-week-old female C57BL/6 mice were purchased from Japan SLC Inc. (Shizuoka, Japan) and kept in our specific pathogen-free animal facility for 6 weeks. The mice were housed in plastic cages with wood-chip bedding and fed commercial pellets (CRF-1; Charles River Japan Inc., Kanagawa, Japan) and tap water *ad libitum*. All animal experiments were conducted with the approval of the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

Sampling of peyer's patches: Small intestines were obtained from three female mice at 10 weeks old of age. From each intestine, the most proximal PP, the most distal PP and the PP nearest to the midpoint between the pylorus and the ileocecal junction were divided under a magnifier. The divided PPs were immersed in RNA*later*™ (QIAGEN GmbH, Hilden, Germany) until isolation of total RNA.

Extraction of RNA: Total RNA was extracted from each PP by using the RNeasy mini kit (QIAGEN GmbH) according to the manufacturer's instruction. The concentration and quality of the extracted RNA were analyzed using a spectrophotometer (Nanodrop, ND-1000; Asahi Techno Glass Corp., Tokyo, Japan) and the Agilent 2100 Bioanalyzer with RNA 6000 Nano Assay Kit (Agilent Technologies Inc., Santa Clara, CA, USA). The extracted RNA samples were kept in a deep freezer until use.

Microarray analysis: Microarray analysis was performed using OpArray™ Mouse V4.0 (Operon Biotechnologies GmbH, Köln, Germany) according to the manufacturer's recommended protocol. In brief, total RNA (1 μg from each sample) was amplified as amino-allyl labeled antisense RNA (aaRNA) using the Amino Allyl MessageAmp™ II kit (Ambion, Austin, TX, USA) for each sample. Further aaRNA of the reference sample (equally pooled from all proximal PP aaRNA samples) and of each experimental sample was labeled with cyanine 3 (Cy3) and cyanine 5 (Cy5), respectively. Cy3-labeled reference probes and Cy5-labeled sample probes were mixed and hybridized to microarrays for 14-16 h. Subsequently, the washed and dried microarray slides were scanned using a microarray scanner (ScanArray lite; PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA, USA). The fluorescence intensity of the sample probes (Cy5) and the reference probes (Cy3) was quantified for each spot on each slide. These data sets were globally normalized using the QuantArray software (PerkinElmer Life and Analytical Sciences Inc.) to obtain median gene signal values.

Statistical analysis: The fluorescence intensity of each spot was corrected as follows:

$$C_s = N_s/N_r * Ave_r$$

Where:

C_s = Corrected value of each sample (Cy5) spot on each slide
N_s = Normalized value of each sample (Cy5) spot on each slide
N_r = Normalized value of each reference (Cy3) spot on each slide
Ave_r = Average of normalized value of each reference (Cy3) spot on all slides

After subtracting the background value, the corrected fluorescence intensity of each spot was converted into a log2 scale. Statistical analysis of each spot was performed using one-way analysis of variance (ANOVA). $p<0.01$ was considered statistically significant. The genes showing significantly different expression levels among the regions were classified according to their known biological functions by using the Database for Annotation, Visualization and Integrated Discovery (DAVID) 2008 (Dennis *et al.*, 2003; Huang *et al.*, 2009). Only the genes classified as immune system process were statistically analyzed using multiple comparisons (Tukey's method).

RESULTS AND DISCUSSION

Statistical analysis of the data revealed that among the proximal, middle and distal PPs, the expressions of 187 genes out of approximately 25,000 genes and 38,000 gene transcripts on the OpArray Mouse V4.0 slides were significantly different ($p<0.01$). These genes were investigated for their involvements in biological functions (GOTERM_BP_1) by DAVID 2008 (Dennis *et al.*, 2003; Huang *et al.*, 2009). The findings were as follows: 93 genes were involved in cellular process; 91, in metabolic process; 30, in biological regulation; 29, in localization; 28, in the establishment of localization; 26, in developmental process; 25, in response to stimulus; 20, in multicellular organismal process; 16, in gene expression; 6, in immune system process; 5, in growth and 3, in multi-organism process. Further, 37 genes were not related to any of the functions mentioned above and 13 genes were not annotated with GO biological process terms (Table 1). Then, the expression of the 6 genes involved in immune system process was statistically compared among the regions (Table 2). The expression of histocompatibility 2, q region locus 1 (*H2-Q1*) gene which is associated

Table 1: Function-based classification of the genes expressed differently among the PPs of different regions

Term	No. of genes identified
Cellular process	93
Metabolic process	91
Biological regulation	30
Localization	29
Establishment of localization	28
Developmental process	26
Response to stimulus	25
Multicellular organismal process	20
Gene expression	16
Immune system process	6
Growth	5
Multi-organism process	3
None of above	37
Not annotated	13
Total	187

Table 2: The expression of the genes related with immune system process among the PPs of different regions (Average±Standard deviation of 3 mice)

Gene symbol	Gene name	Corrected fluorecense intensity (Ave.±S.D.) Proximal (P)	Middle (M)	Distal (D)	ANOVA p-value	Multiple comparizons P vs M	P vs D	M vs D	Main functions (GO number and GO term)
H2-Q1	Histocompatibility 2, q region locus 1	8.06±1.18	0.00±0.00	0.00±0.00	9.35E-06	***	***		GO: 0006955: immune response GO: 0019882: antigen processin and presentation
Il18	Interleukin 18	11.64±0.45	10.31±0.49	8.64±0.89	3.69E-03		**	*	GO: 0006955: immune response GO: 0042088: T-helper 1 type immune response GO: 0042104: positive regulation of activated T cell proliferation
Ang4	Angiogenin, ribonuclease a family, member 4	11.90±0.63	13.88±0.29	13.42±0.40	4.71E-03	**	*		GO: 0019731: antibacterial humoral response
H2-T22	Histocompatibility 2, t region locus 22	1.64±2.83	7.30±0.93	0.09±0.16	5.00E-03	*		**	GO: 0006955: immune response GO: 0019882: antigen processing and presentation
Ccl6	Chemokine (c-c motif) ligand 6	6.39±1.07	9.04±0.38	8.59±0.39	7.06E-03	**	*		GO: 0006955: immune response
Ada	Adenosine deaminase	12.84±0.39	12.44±0.08	11.45±0.46	7.51E-03		**	*	GO: 0002314: germinal center B cell differentiation GO: 0002636: positive regulation of germinal center formation GO: 0002686: negative regulation of leukocyte migration GO: 0002906: negative regulation of mature B cell apoptosis GO: 0006955: immune response GO: 0019731: antibacterial humoral response GO: 0019882: antigen processing and presentation GO: 0030890: positive regulation of B cell proliferation GO: 0042088: T-helper 1 type immune response GO: 0042110: T cell activation GO: 0045580: regulation of T cell differentiation GO: 0048541: Peyer's patch development GO: 0050862: positive regulation of T cell receptor signaling pathway

*: p<0.05, **: p<0.01, ***: p<0.001

with antigen processing and presentation via major histocompatibility complex (MHC) class I, was higher in the proximal PPs than in the middle and distal PPs. The interleukin-18 (*Il18*) gene and the adenosine disaminase (*Ada*) gene were expressed at higher levels in the proximal and middle PPs than in the distal PPs. The former gene encodes a cytokine associated with the T-helper 1-type immune response. The deficiency of the latter gene is associated with severe combined immunodeficiency (Aldrich *et al*., 2000). The angiogenin, ribonuclease A family, member 4 (*Ang4*) gene and the chemokine (c-c motif) ligand 6 (*Ccl6*) gene were expressed at higher levels in the middle and distal PPs than in the proximal PPs. The former gene is associated with antimicrobial immune responses (Hooper *et al*., 2003). The latter gene encodes a murine CC chemokine that usually attracts macrophages (La Fleur *et al*., 2004). Further, the expression of the histocompatibility 2, T region locus 22 (*H2-T22*) gene which is associated with antigen processing and presentation, was higher in the middle PPs than in the proximal and distal PPs. Of the 187 genes that had different expression levels in the proximal, middle and distal PPs of the mouse small intestine, 6 were related to immune system process. However, no significant differences were found in the expression of most of the cytokines, chemokines and their receptors, except for interleukin-18 and chemokine (c-c motif) ligand 6. In addition, no significant differences were found in the expression of antigen receptors, co-receptors and their ligands on the cell surface, except in the case of histocompatibility 2, q region locus 1 and histocompatibility 2, T region locus 22. These findings suggest that the regional differences among the PPs in terms of the immunological activation status or immunological functions may be subtle or not obvious. This may be partly because the PPs consisted heterogeneous cell population, such as T cells, B cells, macrophages, dendritic cells, M cells and so on. Such heterogeneity may have rendered the differences unclear or insignificant. However, the intestines are exposed to a huge amount of antigens and the intestinal immune system is rather suppressed in the normal state (Mayer, 2003). Oral tolerance is one of the suppression mechanism adopted against intestinal antigens (Mayer, 2003). This may also explain why obvious differences were not noted among the PPs obtained from different regions in the normal state. In fact, different regional responses in PPs have been reported in mice after inoculation with heat-killed probiotic bacteria (Yoshikawa *et al*., 2009) and after oral infection with *Cryptosporidium parvum* (Boher *et al*., 1994).

CONCLUSION

Although the regional differences in the mouse PPs were not obvious in terms of immunological activation status and immunological functions, the regional differences in other functional properties of the PPs should be examined in future studies.

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