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Development of a functional cDNA array for evaluation of the Th1/Th2 balance

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Abstract

The immune balance controlled by $CD4^+$ helper T cell subsets (T helper 1 (Th1) and T helper 2 (Th2)) is crucial for immunoregulation and its imbalance causes various immune diseases including infections, allergic disorders and autoimmune diseases. Therefore, it is of great importance to develop a system of diagnosing Th1/Th2 imbalances for curing immune diseases. Here we developed a functional cDNA array filter useful for assessing the Th1/Th2 balance in mice. To overcome the disadvantages of conventional microarrays carrying thousands of genes, we prepared an array filter containing 40 Th1-specific and 32 Th2-specific genes, which were selected from over 8700 genes based on (i) the specificity of expression in Th1 or Th2 cells and (ii) an expression level which is high enough for detection using a DNA array. This array filter provided a prompt and precise evaluation for the skewing of the Th1/Th2 balance combined with our calculation algorithm. The bias toward Th1 or Th2 was evaluated visually at a glance by aligning the genes on the filter. Moreover, we succeeded in evaluating the skewing of the Th1/Th2 balance in vivo during acute graft versus host disease (GVHD). Thus, this array filter will provide a novel tool for evaluation of the Th1/Th2 balance in a variety of immune diseases.

Keywords: Th1/Th2 balance; DNA array; Diagnosis; Gene expression

1. Introduction

$CD4^+$ T helper cells are classified into two subsets, T helper 1 (Th1) and T helper 2 (Th2) cells, according to their cytokine production profile [1]. Th1 cells play a critical role for the regulation of cellular immunity through the secretion of IL-2, IFN-γ and TNF-β. On the other hand, Th2 cells regulate humoral immunity by producing IL-4, IL-5, IL-6, IL-10 and IL-13. It is now widely accepted that the immune balance regulated by these Th1- and Th2-derived cytokines (Th1/Th2 balance) is critically important in immunoregulation, and its imbalance becomes the cause of the immune diseases [2–5]. The critical role of Th1 or Th2 cells for inducing immune diseases has been demonstrated by many animal experimental models. For instance, an excessive acceleration of Th1 immunity causes the onset of liver injury [6,7], GVHD [8], diabetes [9] and multiple sclerosis [10], whereas allergic diseases such as asthma [11] are derived from dysregulated Th2 responses. Recently, it became possible to modulate immune balance through controlling the function of innate immune effector cells such as dendritic cells (DC) and NKT using CpG oligonucleotides [12] or α-galactosylceramide (α-GalCer) [13]. Especially, CpG, a ligand of toll-like receptor 9 (TLR9) has been applied to clinical trial of Th2-dependent allergic diseases because it can induce a strong type1-immunity in vivo [12]. Such immunomodulator with a capability of controlling Th1/Th2 balance will become a novel strategy for therapeutic approach to immune diseases. However, we also have to take care about

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the adverse effect of such immunomodulating drugs because Th1/Th2 balance is genetically controlled. In the case of IL-12 clinical trial, the responsiveness to IL-12 was different in each patient and some patients suffered from lethal toxic effect [14]. Therefore, diagnosis of Th1/Th2 balance in each patient is essential for the application of immune balance regulating drugs. The diagnosis of Th1/Th2 balance is also useful for early detection of recurrence or cure of immune diseases. For this purpose, we developed a DNA array for evaluation of the Th1/Th2 balance.

DNA arrays are useful for determining the expression levels of a number of genes at once. Different methods have been developed which use either solid glass/plastic or a nylon membrane to support DNA. Furthermore, the DNA to be mounted can be different. Some arrays are mounted with oligodeoxynucleotides specific to each gene, while others use cDNA fragments. We chose a cDNA array based on a nylon membrane for the following reasons: (1) the number of genes to be mounted, i.e. the number of genes which show Th1/Th2 specificity is limited and (2) results with membrane filters are more reproducible than those with solid bases. We also chose a chemiluminescence-based method of detection, as it is broadly applicable and does not require any specific facilities or equipment. To overcome the low sensitivity of the method, we amplified the cDNA probe using a PCR-based approach. In this way, we are able to detect signals at a sensitivity equivalent to that of the methods employing radioisotopes.

There are several commercial DNA arrays containing immunologically important genes, and some researchers have reported differences in gene expression between Th1 and Th2 cells using the DNA array method [15–18]. The goal of this study is to develop a DNA array that can be used to measure the expression level of Th1- or Th2-specific genes, and apply it for the evaluation of the Th1/Th2 balance in vivo. By choosing a limited number of genes and developing a scoring method, we could easily and reliably evaluate the Th1/Th2 balance in a mouse immune disease model.

2. Materials and methods

2.1. Generation of Th1 and Th2 cells

Th1 and Th2 cells were induced from DO11.10 TCR transgenic mice (maintained on the BALB/c background, donated by Dr. K.M. Murphy). $CD4^+CD45RB^+$ naive T cells were isolated from nylon-passed spleen cells of DO11.10 TCR-transgenic mice by cell sorting using a FACS Vantage instrument (BD Bioscience, Mountain View, CA). Purified $CD4^+CD45RB^+$ cells were stimulated with 10 μg/ml $OVA_{323\text{-}339}$ peptide (donated by Fujiya, Hadano, Japan) in the presence of mitomycin C-treated BALB/c spleen cells, 20 U/ml IL-12 (donated by Genetics Institute, MA), 1 ng/ml IFN-γ (BD PharMingen, San Diego, CA), 50 μg/ml anti-IL-4 mAb (11B11, ATCC, Rockville, MD) and 20 U/ml IL-2 (donated by Shionogi Pharmaceutical, Osaka, Japan) for Th1 development. Th2 cells were induced from the same naive Th cells in the presence of 1 ng/ml IL-4 (PEPROTECH Inc., Rockyhill, NJ), 50 μg/ml anti-IFN-γ mAb (R4-6A2, BD Pharmingen), 50 μg/ml anti-IL-12 mAb (C15.1 and C15.6, provided by Dr. G. Trinchieri, Wister Institute of Anatomy and Biology, Philadelphia, PA), and 20 U/ml IL-2. After 48 h, cells were restimulated with $OVA_{323\text{-}339}$ under the same conditions, and used at 12 days of culture. Before RNA extraction, some cells were stimulated with immobilized anti-CD3 mAb for 2 h at 37 °C.

2.2. GEM microarray analysis and semi-quantitative RT-PCR

Total RNA was isolated from Th1 or Th2 cells and mouse spleen cells by the acid guanidinium-phenol-chloroform (AGPC) method using ISOGEN (Nippon gene, Toyama, Japan). The $poly(A)^+$ mRNA was purified from total RNA by incubation with oligo-dT magnetic beads (Toyobo Co., Osaka, Japan). Microarray analyses were performed using the GEM microarray (Incyte Genomics, Palo Alto, CA) with $poly(A)^+$ RNAs from anti-CD3 mAb-stimulated or unstimulated Th1 and Th2 cells. The microarray was spotted with about 8700 EST clones.

For semi-quantitative RT-PCR, cDNAs were synthesized by reverse transcription of 1 μg of total RNA at 42 °C for 50 min, using 200 U of Superscript II (Life Technologies, Rockville, MD) and $oligo(dT)_{12\text{–}18}$. PCRs were performed to amplify each cDNA and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA as a control using Taq DNA polymerase (Promega, Madison, WI) or KOD dash (Toyobo Co.). Reactions with Taq DNA polymerase were performed in a thermal cycler for 25–35 cycles (denaturation: 30 s, 94 °C; annealing: 30 s, 60 °C; extension: 1 min, 72 °C). Reactions with KOD dash were performed for 25–35 cycles (denaturation: 20 s, 98 °C; annealing: 2 s, 60 °C; extension: 30 s, 72 °C). The efficiency of each reverse transcription reaction was confirmed by the PCR for GAPDH. For quantification, PCR products of different cycles were electrophoresed on agarose gels and visualized by ethidium bromide staining. The gel images were incorporated by Densitograph (ATTO, Tokyo, Japan) and each band was quantified using Lane & Spot Analyzer (ATTO). The quantification for each gene was done at a point at which no saturation of the PCR was evident.

2.3. Production of cDNA array filters

A cDNA fragment of about 500 bp was amplified by PCR using an unique primer set for each gene. Primers were selected so that the amplified cDNA fragment does not have high homology to other genes in the database. Each PCR fragment was cloned into pGEM-T Easy vector (Promega) and verified by DNA sequencing with a BigDye™ Terminator DNA sequencing kit (Applied Biosystems, Foster City, CA) and ABI310 Genetic Analyzer (Applied Biosystems). Each