

## The kinetics of IL-4 and IFN- $\gamma$ gene expression in Mice after Trichosansin immunization

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### ABSTRACT

Trichosanthin (TCS) is a potent allergen to mice. According to our previous experiments, it could bring out the IgE response to ovabumin (OVA) if TCS was given one day before OVA immunization, while OVA alone could not induce IgE to it. In this work, the kinetics of interleukin 4(IL-4) and interferon  $\gamma$  (IFN- $\gamma$ ) gene expression in the mesenteric lymph node (MLN) of TCS-immunized mice was investigated using a semi-quantitative RT-PCR method. It indicated that TCS induced significant IL-4 gene expression and the peaks of IL4 gene expression were on day one after TCS immunization in both primary and secondary response. In contrast, the IFN- $\gamma$  gene expression was suppressed. Furthermore, the IL-4 gene expression in the secondary response was lower than that in the primary response. Thus the presence of IgE memory B cells were studied. Results showed that the amount of mature IgE mRNA arose significantly and rapidly one day after TCS restimulation, while in the MLN of the mice primed 30 days before and without boost, it was almost as the same amount of the unimmunized control. These findings suggest the existence of the IgE memory B cells in the mice after the primary TCS immunization.

**Key words:** *Trichosanthin, IL-4, IFN- $\gamma$ , IgE.*

### INTRODUCTION

Trichosanthin is a plant protein extracted from a Chinese medicinal herb. It has been shown to have effect in abortion induction, and anti-HIV and anti-cancer effect

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as well. Previously we reported[1] that TCS is a potent allergen and we found when TCS co-immunized with ovabumin, it would also bring out the IgE response to OVA, which induced no IgE response when it was used alone. However, this "helping" effect of TCS was under a narrow time window. It only worked when TCS was given one day ahead of OVA immunization. Since IL-4 plays a pivotal role in IgE switch, we suppose that TCS induces profuse production of IL-4. Due to "bystander" effect, it also drives the OVA-specific B cell clones to switch to IgE secretion. In this paper semi-quantitative RT-PCR is used to study the expression kinetics of IL-4 and IFN- $\gamma$  genes. Furthermore during our preliminary kinetic studies, we found that IL-4 expression in secondary response was significantly lower than that in primary response. We suppose that it may be due to the presence of a number of IgE memory B cells. They proliferate to support the secondary IgE response and require little IL-4. In this study the IgE memory B cells in both primary and secondary responses were also studied.

## MATERIALS AND METHODS

### *Materials*

Mice: C57BL/6J mice, female and 12 weeks of age.

Antigen: crystalized TCS in Trichosanthin Injection Solution produced by Jin-San Pharmaceutical Factory, 1.2 mg/ml, was used as immunogen.

Immunization: TCS was diluted in PBS and adjusted to 10 mg/ml, each mouse was intraperitoneally injected with 0.5 ml antigen solution.

### *Methods*

#### *RNA preparation and reverse transcription*

Total RNA was extracted from MLN by the acid guanidinium isothiocyanate method, as described elsewhere[2]. RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (GIBCO-BRL) in the presence of 1 mM of each dNTP (dATP, dGTP, dCTP, dTTP), 5  $\mu$ M random hexamer primers, 0.1 M DTT, 5  $\times$  first strand buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl<sub>2</sub>, pH8.0). The RT mix was incubated at 37  $^{\circ}$ C for 1 h. Samples were stored at -20  $^{\circ}$ C until use.

#### *PCR and quantitative analysis*

Semi-quantitative PCR was carried out according to Chelly[3] with modification. PCR were performed in 50  $\mu$ l reaction volumes, which contained: 5  $\mu$ l of 10  $\times$  reaction buffer, 4  $\mu$ l of 2.5 mM dNTPs, 1  $\mu$ l of each appropriate sense and antisense primer (25 pmol/ $\mu$ l), 6  $\mu$ l cDNA sample, 2 units Of Taq DNA polymerase (SINO-AMERICAN BIOTECH). The primers for each target gene were added to the tube before the first cycle and the second primer set for house-keeping gene  $\beta$ -actin was added by the "primer-dropping" method after ten cycles had been completed[4]. After an initial incubation at 94  $^{\circ}$ C for 5 min, temperature cycling was initiated with each cycle as follows: 1)94  $^{\circ}$ C for 1 min (denaturation); 2) 55  $^{\circ}$ C to 60  $^{\circ}$ C (depending on the target gene to be amplified) for 1 min (annealing); 3)72  $^{\circ}$ C for 1 min (extension). PCR amplifications were performed in a Temperature Cycling System (CEL-BIO). PCR products were collected at different cycles as shown in figures (see Results) and up to 34 cycles were performed. 20  $\mu$ l of PCR products were electrophoresed through the 6% polyacrylamide gels, stained with ethidium bromide and photographed. Appropriate bands were scanned and quantitated from the negative film using the Complete Gel Documentation and Analysis System (UVP) with Gel work 3.01 program. For each

gene product, the optimal numbers of cycles were determined. The determination of cycle numbers is to assure the cDNA achieved after cycling could be detected, but its concentration is well below the saturating condition, which permits to do a kinetic study instead of entering the plateau stage immediately. In this way, the rate of amplification was exponential for these cycles.

### Primers used for the PCR

Primers were chosen according to references as follows:

IL-4:	sense primer:	5'-GAATGTACCAGGAGCCATATC-3'	385 bp [5]
	antisense primer:	5'-CTCAGTACTACGAGTAATCCA-3'	
IFN- $\gamma$ :	sense primer:	5'-AACGCTACACACTGCATCTTGG-3'	237 bp [5]
	antisense primer:	5'-GACTTCAAAGAGTCTGAGG-3'	
IgE:	sense primer:	5'-AAGGCACTGCTTCCATGACC-3'	512 bp [6]
	antisense primer:	5'-GGAGGTGACCTTGCAGGTGA-3'	
$\beta$ -actin:	1) sense primer:	5'-GTGGGCCGCTCTAGGCACCAA-3'	540 bp [7]
	antisense primer:	5'-CTCTTTGATGACACGCACGATTTTC-3'	
	2) sense primer:	5'-GTGGGCCGCTCTAGGCACCAA-3'	245 bp [6]
	antisense primer:	5'-GGAGGTGACCTTGCAGGTGA-3'	

The 540 bp fragment of  $\beta$ -actin was used for the target cytokines IL-4 and IFN- $\gamma$ . Because the PCR product of IgE constant region was 512 bp, which would be too close to the  $\beta$ -actin in the gel, we chose another set of primers (set 2) for the internal standard  $\beta$ -actin for the target IgE.

## RESULTS

### Kinetics of IL-4 and IFN- $\gamma$ gene expression in MLN of TCS immunized mice

RNA was prepared from MLNs of mice killed on different days after TCS immunization. RT-PCR was used to amplify each of the target genes and internal standard  $\beta$ -actin. Each size of the fragments was determined by the DNA molecular marker (Fig 1). The final products of PCR after electrophoresis shown on negative film were scanned and analyzed (only part of the results are shown here, Fig 2a, Fig 3a for day 0 and day 31 respectively). Results are expressed as a percentage of  $\beta$ -actin cDNA copies, which was run in parallel for each sample.

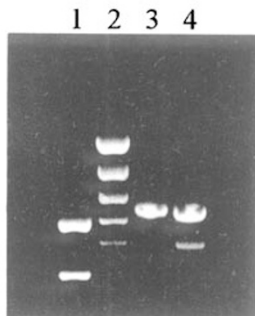


Fig 1. PCR production of target gene and internal standard  $\beta$ -actin.

Lane 1: IgE (512bp) and  $\beta$ -actin (245bp)

Lane 2: DNA markers (154bp, 994bp, 659bp, 515bp, 377bp, 237bp)

Lane 3:  $\beta$ -actin (540bp) and IFN- $\gamma$  (237bp)

Lane 4:  $\beta$ -actin (540bp) and IL-4 (385bp)

In Fig 2, day 0 represents the mRNA level expressed in the MLN before priming. IL-4 gene expression was increased significantly as early as one day after first immu-

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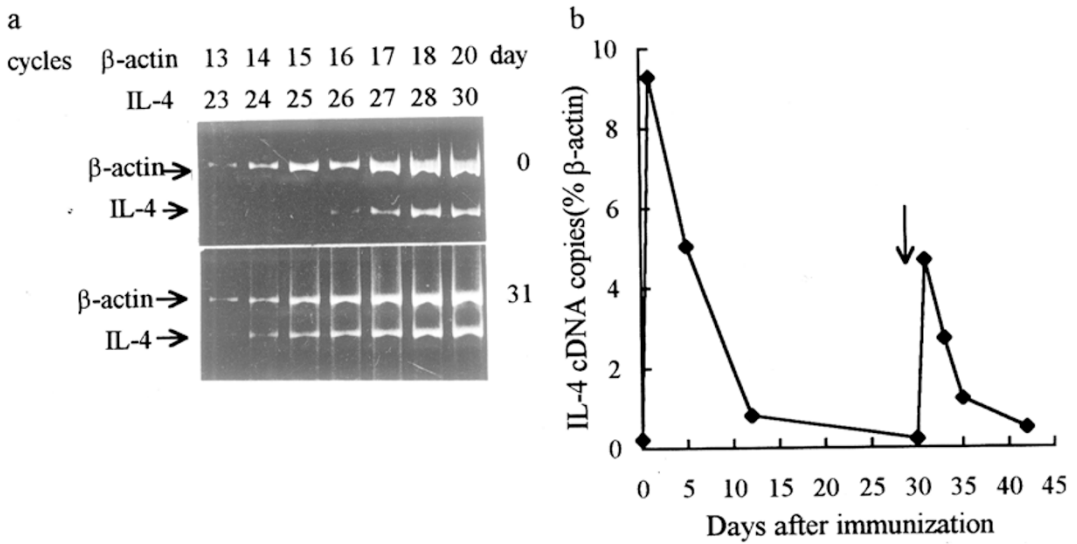


Fig 2. Expression of IL-4 gene in mesenteric lymph node cells of the TCS immunized Mice  
 (a) PCR products of IL-4 ended at different cycles were electrophoresed through 6 % polyacrylamide gels (only day 0 and day 31 are shown here).  
 (b) Kinetics of IL-4 gene expression. The arrow indicates a booster injection with 5  $\mu$ g TCS given on day 30.

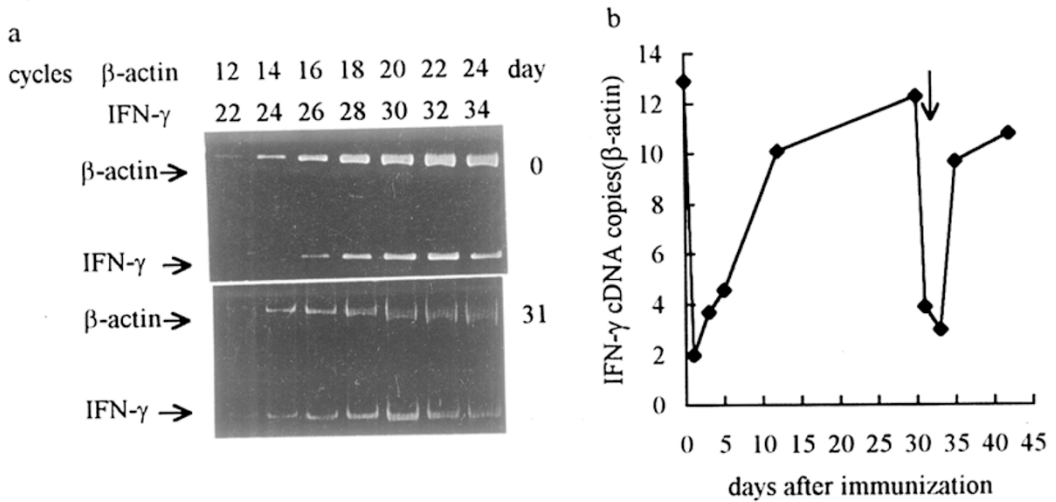


Fig 3. Expression of IFN- $\gamma$  gene in mesenteric lymph node cells of the TCS immunized Mice  
 (a) PCR products of IFN- $\gamma$  ended at different cycles and electrophoresed through 6 % polyacrylamide gels (only day 0 and day 31 are shown here).  
 (b) Kinetics of IFN- $\gamma$  gene expression. The arrow indicates a booster injection with 5  $\mu$  g TCS given on day 30.

nization (from 0.34%  $\beta$ -actin to 10.44%  $\beta$ -actin), and then reduced with days until to normal level (on day 30). The IL-4 gene expression increased again one day after boosting, but the percentage was lower than that after first immunization.

In contrast, the normal level (day 0) of IFN- $\gamma$  gene expression (Fig 3) is pretty high (12.9%  $\beta$ -actin). TCS immunization suppressed the IFN- $\gamma$  gene expression, resulting in a decreased amount below unimmunized controls. From day 2, the IFN- $\gamma$  mRNA level increased gradually until to normal level. The changes in IFN- $\gamma$  gene expression after boost showed the similar pattern.

IgE gene expression before and after TCS restimulation

We also used quantitative RT-PCR method to study the IgE gene expression in order to investigate the existence of memory B cells for IgE. Fig 4 shows that the IgE mRNA expression in MLN taken from the mice one day after boost was increased by approximately 7 fold over that before the boosting (from 3.4%  $\beta$ -actin to 20.6%  $\beta$ -actin), which was at the same level as control (3.6%  $\beta$ -actin).

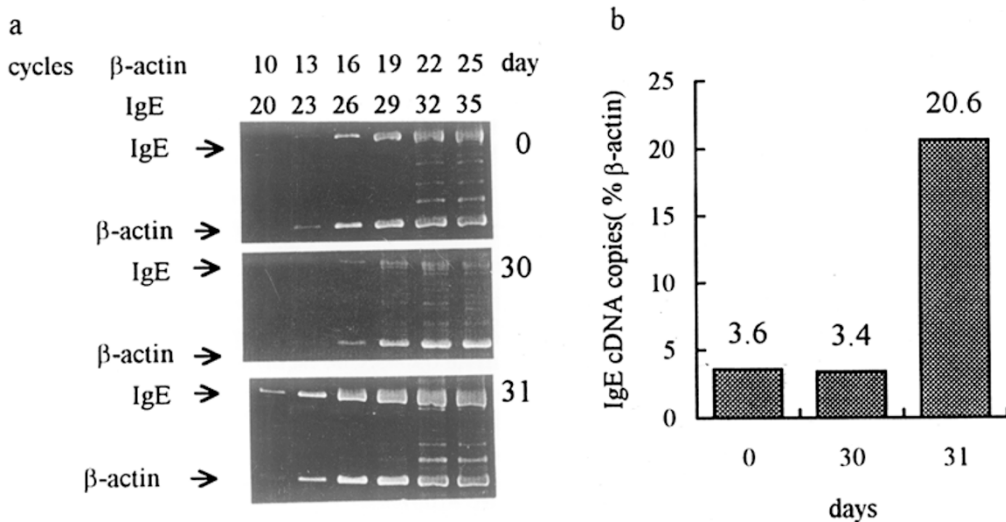


Fig 4. IgE gene expression before and after TCS restimulation.  
 (a) PCR products of IgE were electrophoresed through 6% polyacrylamide gels.  
 (b) IgE gene expression. Day 0 represents the level expressed in the MLN of unimmunized control. Day 31 represents one day after TCS boost.

DISCUSSION

IgE response is mediated by helper T cells. According to the patterns of the cytokines secreted they can be classified into two catagories, Th1 and Th2 cells. Th1 cells make larger amount of IL-2, IFN- $\gamma$  TGF- $\beta$ , and TNF- $\alpha$ , while Th2 cells make

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more IL-4, IL-5, IL-6 and IL-10. As for their role in the regulation of IgE isotype, it is commonly regarded Th2 cells are capable of inducing significant IgE responses, whereas none of the Th1 clones tested could induce detectable IgE production. In fact the induction of IgE by Th2 but not by Th1 can be entirely explained by their difference in IL-4 and IFN- $\gamma$  production. Th1 clones can induce good IgE response if IL-4 and anti-IFN- $\gamma$  antibodies are added in cultures[8]. Since IL-4 and IFN- $\gamma$  are two most important cytokines in the regulation of IgE responses, in this paper we focused on the kinetics of their gene expression instead of analysis of the Th cell types involved.

### *Significant expression of IL-4 gene with a peak one day after TCS immunization*

In the present study, it indicates that TCS has the potent effect to induce the expression of IL-4 gene. Especially in the primary response it was much higher over the expression amount of the background value. Furthermore in both primary and secondary response the peaks of IL-4 expression were all on day one after TCS immunization and then gradually went down. The RNA expression of IL-4 may reflect the amount of secreted IL-4, since according to the study of Sun[9], the relative IFN- $\gamma$  mRNA expression determined by quantitative PCR parallels the amount of secreted IFN- $\gamma$  protein. Thus this IL-4 expression pattern seems that it may support our previous view that TCS induced IL-4 may help the OVA-specific B cells switch to IgE. In our previous study[1], OVA IgE response did not occur when TCS was given 3-5 days ahead, or one day after OVA immunization, or TCS and OVA given at the same time. Only TCS given one day ahead of OVA immunization was worked. We suppose on the day of OVA immunization, in the micro-environment where the OVA-primed B cells located, adequate amount of IL-4 induced by TCS immunization one day ahead is already present around and helps the OVA-primed B cells to switch to IgE production.

### *The existence of IgE memory B cells*

It can be seen from the Fig 2 that, to our surprise, the IL-4 gene expression in the primary response is significantly higher than that in the secondary response. We repeated this experiment several times and got the same results. Therefore during the primary response the IgE response was lower but the IL-4 expression was higher, while during the secondary response the IgE response was higher but the IL-4 expression was lower. That probably signified that less IL-4 was needed during the secondary response. Since IL-4 plays a pivotal role in IgE switch, those switched IgE memory B cells, although they proliferate rapidly and secrete large amount of specific IgE in response to restimulation by antigen, may require little IL-4. It was also shown in Haruna's paper that IL-4 was not required in the secondary IgE response[10]. From Fig 4 it can be seen that one day after TCS restimulation (day 31), in the MLN of the boosted mice the mRNA for IgE molecules arise rapidly. Only

IgE memory B cells can respond so rapidly. According to Takahama[6], during the primary response IgE could be detected only on day 7 after antigen priming. This suggests the existence of IgE memory B cells to TCS. If so, the TCS-specific IgE memory B cells were generated during the primary response.

### IFN- $\gamma$ expression versus IL-4 expression

The down regulation of IFN- $\gamma$  gene expression in TCS-IgE response is just as we have expected. It is commonly regarded that IFN- $\gamma$  is suppressed during IgE response. IL-4 is reported to suppress IFN- $\gamma$  production by human lymphocytes, probably by inhibiting IFN- $\gamma$  gene transcription[11]. CD8<sup>+</sup> T cell suppression may be an alternative possibility[12]. It is interesting to note that IFN- $\gamma$  expression was also significantly reduced one day after TCS immunization in both primary and secondary response, and then went up. It seems no big differences in the reduction between the primary and secondary response. The down and up of IFN- $\gamma$  expression was just opposite to the up and down of IL-4 expression. It reflects the meticulous regulation of different cytokine genes both temporally and spatially during the immune responses.

### ACKNOWLEDGEMENT

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