The Journal of Kamakura Women's University, No.14, pp.13-24, March 2007

原著論文

Development of In Vitro Screening System for Photo Allergen Using Splenic Cells from Photosensitized Mice

Yamazaki, Shunsuke・Yamaguchi, Mayu・Andou, Hirofumi

光感作マウスの脾細胞を用いた光アレルギー反応のIn Vitroスクリーニング法の開発

山﨑　俊介（管理栄養学科・助教授）・山口　真由（管理栄養学科・助手），
安藤　博文（初等教育学科・教授）

Abstract

Splenic cells from mice that had induced contact photosensitivity (CPS) by treatment with 3, 3', 4', 5-tetrachlorosalicylanilide (TCSA) were transplanted to untreated naive mice. The CPS reaction was elicited with TCSA in the auricle of the mice 24 hours after the cell transplant. Ear-swelling, equivalent to that of the mice photosensitized with TCSA, was observed. CPS reaction was elicited in photosensitized mice either by applying UVA-irradiated photo hapten (TCSA) or by applying TCSA, then irradiating with UVA after application. 24 hours later, ear-swelling was checked and cytokine expression in the lesion was analyzed using RT-PCR. Significant ear-swelling and IL-4mRNA expression were observed in both groups. These test results indicate that the TCSA-triggered CPS can be induced by cell transplantation, and that the CPS can be elicited by UVA-radiated TCSA. CPS reaction was then studied in vitro with the total spleen cells from the mice photosensitized by TCSA, using Th2 cytokine production as the indicator. The study was conducted on 3 groups of cells. For group Ⅰ, photo hapten (TCSA) was added to the cells and then UVA irradiation is performed (regular photosensitive group); for group Ⅱ, UVA-treated photo hapten (TCSA) was added to the cells; for group Ⅲ, photo hapten (TCSA) was added to the UVA -treated cells. For each group, the cytokine in culture supernatant was measured by ELISA. IL4 and IL5 production increased in group Ⅰ and Ⅱ supporting the previously obtained data suggesting that Th2 cytokine plays an important role in CPS reaction. Our study indicates that in vitro screening of photo allergen using Th2 cytokine production as an indicator is effective to some extent.

Keywords : Contact photosensitivity ; Cytokines ; Spleen cells
キーワード：光接触過敏症、サイトカイン、脾細胞

1. Introduction

CPS is a cutaneous allergic reaction induced and elicited by skin application of a photo allergen plus irradiation of the skin with ultraviolet A light (UVA).[1-3] CPS to TCSA and other known photo allergens has been successfully induced in mice [4] and is genetically controlled. [5] Murine CPS to a photo hapten, TCSA, is a delayed-type hypersensitivity reaction involving both positive and negative immunologic pathways that are restricted respectively by I-A and I-E molecules on APC. [5,6] The responsiveness of CPS to TCSA differs between mouse strains and depends especially on the H-2 haplotype and fur color .[7,8] The H-$2^{b,d}$ haplotypes are closely associated with high responders, whereas mice with H-2k are low or non-responders.[7] The hyporesponsiveness of CPS to photo allergens in AKR/n (H-2^{k}) mice was closely associated with the activation / induction of Th2 cytokines at the challenged sites.[9] Thus, studies of CPS to TCSA are well suited to investigate the role of Th2 cells in cutaneous hypersensitivities. In this study, the CPS reaction was studied in vitro with the total spleen cells from the mice photosensitized by TCSA, using Th2 cytokine production as the indicator. In the present study, we compared regular CPS with (1) CPS by cell transplantation (experiment 1), (2) analysis of TCSA-triggered CPS by UV-A-radiated TCSA(experiment 2) and (3) an in vitro CPS reaction with the total spleen cells from the mice photosensitized by TCSA, using Th2 cytokine production as the indicator (experiment 3).

2. Materials and methods

2.1. Animals

Female BALB/c mice were obtained from Clea Japan, Inc. (Tokyo, Japan). They were used at 7 weeks of age. Each group consisted of more than five mice.

2.2. Chemicals

TCSA (Eastman Kodak Co., Rochester , N Y) was used as photoallergic agent.

2.3. Light source

Black light (FL20S-BLB; Toshiba Electric Co., Tokyo, Japan) emitting UVA ranging from 320 to 400 nm with a peak emission at 365 nm was used for irradiation. The light was passed through a pane of 4-mm-thick glass. The energy output of 24, 20-W tubes of black light at a distance of 40 cm was 2.2 mW/cm^2 at 365 nm.

2.4. Sensitization and elicitation of CPS

For sensitization, 100 μl of 1% TCSA , in an olive oil-acetone mixture (1:4) was applied epicutaneously on the shaved back skin of mice, and the site was irradiated with UVA at a distance of 40 cm for 2 h (16J/cm^2 at 365 nm) on days 0 and 1. Mice were challenged on both sides of the earlobe with an a day 5, each mouse application of 40 μl of 0.1% TCSA in an olive-acetone mixture (1:4) followed by UVA irradiation at a distance of 40cm for 2h (16J/cm^2). For experiment 2, mice were challenged on both sides of the earlobe with an a day 5, each mouse application of 40 μl of 0.1% irradiated TCSA (16J/cm^2) in an olive-acetone mixture (1:4). After each measurement, ear thickness was measured 0, 6, 12, 24, 48 and 72h after completion of irradiation.

2.5. Spleen Cell (SC) suspensions

Mice were photosensitized with TCSA plus UVA on days 0 and 1. On day 5, single-cell suspensions were prepared by teasing spleens in RPMI-1640 (GIBCO, Grand Island, NY). In each experiment, more than 90% of the cells were viable as determined by trypan blue dye exclusion.

2.6. Preparation of T lymphocyte

T lymphocyte (TL) was collected from the SC suspensions of the mice 5 days after photosensitization. After filtration through a nylon wool column, cells were suspended in RPMI-1640 using a solution supplemented with 2 mM L-glutamine, 2.5×10^{-5} M 2-mercaptoethanol, 100 units/ml penicillin,0.1 mg/ml streptomycin, and 10% heat-inactivated fetal calf serum.

2.7. Cell transfer experiments

TL cells (1×10^7 cells/0.5ml) from mice that had induced CPS by treatment with TCSA were

transplanted to untreated naïve mice. The CPS reaction was elicited with TCSA in the auricle of the mice 24h after the cell transplant (experiment 1).

2.8. RT-PCR

The study (experiment 2) was conducted on 4 groups. That is to say CPS reaction was elicited in photosensitized mice either by applying TCSA, then irradiating with UVA after application (group Ⅰ) or by applying UVA-irradiated photo hapten (TCSA) (group Ⅱ). Both groups compared with corresponding negative control groups (groupⅢ is UVA alone in elicitation and groupⅣ is TCSA alone in elicitation). Earlobes of mice were removed 0, 12, 24, 48 and 72h after challenge and rapidly homogenized in a lysis buffer. cDNA was synthesized from oligo-dT (Pharmacia Biotechnology AB, Uppsala, Sweden)-primed RNA by incubation at 43 ℃ with M-MLV reverse transcriptase (Gibco BRL, Gaithersburg) and 0.5 M dNTPs (Pharmacia) for 1h. cDNA was amplified by PCR, using oligonucleotide primer specific for β-actin, IL-2, IL-4, IL-5 and IFN-γ. The primers used were as follows: interleukin (IL) -2, 5' primer ATGTACAGCATG-CAGCTCGCATC, 3' primer GGCTTGTTGAGAT-GATGCTTTGACA; IL-4 , 5' primer ATGGGTCT-CAACCCCCAGCTAGT, 3' primer GCTCTTTAG-GCT-TTCCAGGAAGTC; IL-5, 5' primer CTCTAG-TAAGCCCACTTCTA, 3' primer TGATACCT-GAATAACATCCC ; interferon-γ (IFN-γ), 5' primer TGAACGCTACACACTGCATCTTGG, 3' primer CGACTCCTTTTCCGC-TTCCTGAG; and β-actin, 5' primer TGGAATCCTGTGGCATCCATG-AAAC, 3' primer TAAAACGCA GCTCAGTAACAGTCCG. A DNA thermal cycler (Perkin- Elmer Cetus, Norwalk CT) was used for 25 cycles (1 min of denaturation at 94℃ , 2min of annealing at 60℃, and 3 min of extension at 72℃). An aliquot of PCR product was then electrophoresed on 1.5% agarose gels and visualized by ethidium bromide staining.

2.9. SC culture and assay for cytokines

The study(experiment 3) was conducted on 3 groups of cells. For group Ⅰ, photo hapten (TCSA) was added to the cells and then UVA irradiation is performed (regular photosensitive group); for group Ⅱ, UVA-treated photo hapten (TCSA) was added to the cells; for groupⅢ, photo hapten (TCSA) was added to the UVA-treated cells. For each group, the cytokine in culture supernatant was measured by enzyme-linked immunosorbent assay (ELISA) kit (Amersham Pharmacia Biotech).

3. Results

For experiment 1,splenic cells (nylon wool-passed immune T cells) from mice that had induced contact photosensitivity (CPS) by treatment with TCSA were transplanted to untreated naive mice. The CPS reaction was elicited with TCSA in the auricle of the mice 24h after the cell transplant. Ear thickness was measured 0, 6, 12, 24, 48 and 72h after completion of irradiation. Equivalent to that of the mice photosensitized with TCSA, was observed (Fig.1). In both groups, the ear swelling responses of mice reached maximum at 24-48 h after challenge and remained at 72h. These data suggested that the TCSA-triggered CPS can be induced by cell transplantation.

For experiment 2,CPS reaction was elicited in photosensitized mice either by applying TCSA, then irradiating with UVA after application (group Ⅰ) or by applying UVA-irradiated photo hapten (TCSA) (group Ⅱ). Both groups compared with corresponding negative control groups (groupⅢ is UVA alone in elicitation and groupⅣ is TCSA alone in elicitation). 24h later, ear swelling was checked and cytokine expression in the lesion was analyzed. In both groups, the ear swelling responses of mice reached maximum at 24-48h after challenge and remained at 72h (Fig.2). The expression of T-cell cytokine mRNA was examined by RT-PCR in the challenged earlobes of mice . Fig.3 represents PCR bands of each cytokine in CPS to TCSA in both groups Ⅰ and Ⅱ. In both groups, mRNA for Th1 cytokines, IFN-γ and IL-2, were only slightly expressed. Of the Th2 cytokines, IL-4 was clearly upregulated and IL-5 was expressed only

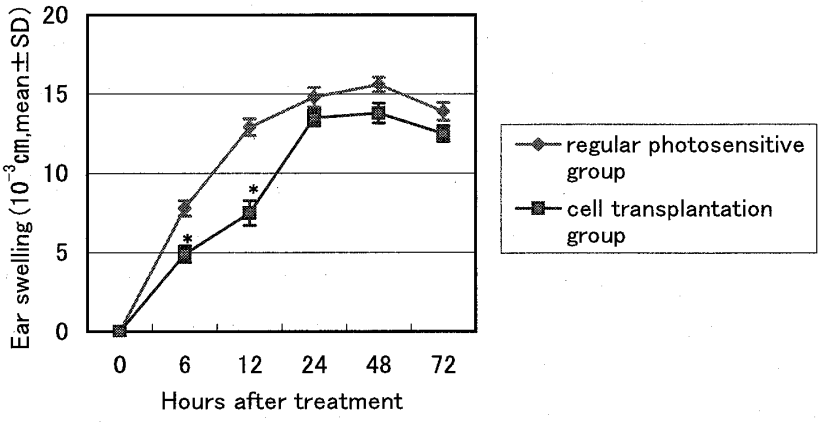

Fig.1. Ear swelling responses of CPS to TCSA in Balb/c mice

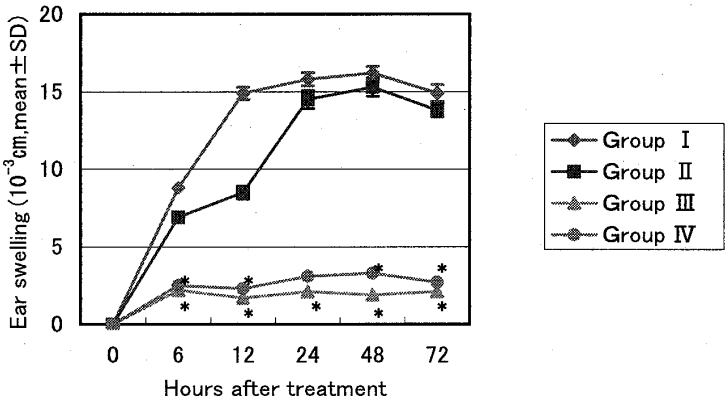

Fig.2. Ear swelling responses of CPS to TCSA in Balb/c mice
Group Ⅰ; regular photosensitive group
Group Ⅱ; applying UVA irradiated photo hapten (TCSA)
Group Ⅲ; UVA irradiated, no application of TCSA
Group Ⅳ; TCSA application, no UVA irradiation

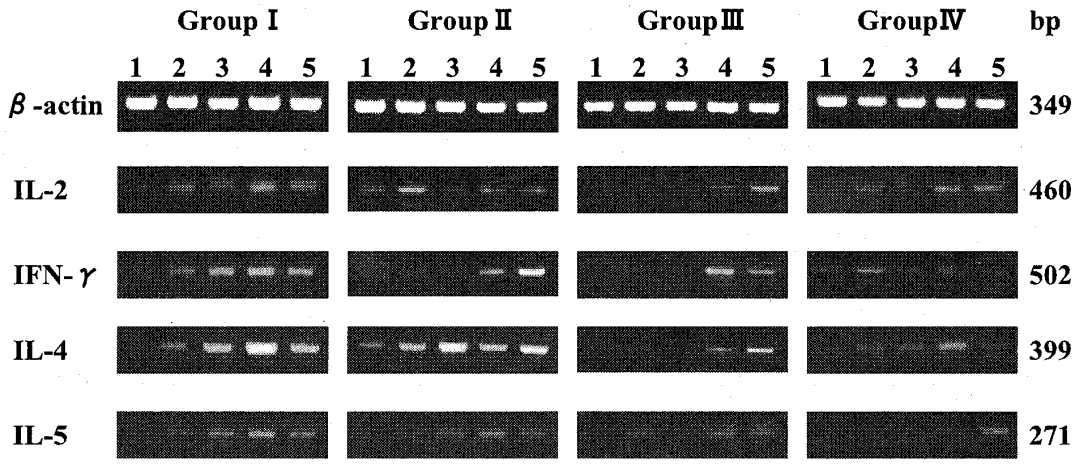

Fig.3. RT-PCR analysis of cytokine mRNA expression in the ear after treatment

slightly. On the other hand, negative control groups (group Ⅲ and group Ⅳ) didn't exhibit an ear swelling response and Th1 and Th2 cytokines were slightly expressed. These data suggested that the CPS can be elicited by UVA-radiated TCSA.

For experiment 3, CPS reaction was then studied in vitro with the total spleen cells from the mice photosensitized by TCSA, using Th2 cytokine production as the indicator as assayed by ELISA. The study was conducted on 3 groups of cells. For group Ⅰ, photo hapten (TCSA) was added to the cells and then UVA irradiation is performed (regular photosensitive group) ; for group Ⅱ , UVA-treated photo hapten (TCSA) was added to the cells; for group Ⅲ , photo hapten (TCSA) was added to the UVA-treated cells. For each group, the cytokine expression in culture supernatant was measured by ELISA. IL-4 and IL-5 production increased in group Ⅰ and Ⅱ (Fig.4-7). These date suggested that Th2 cytokine especially expressed in CPS reaction of lymphoid cells .

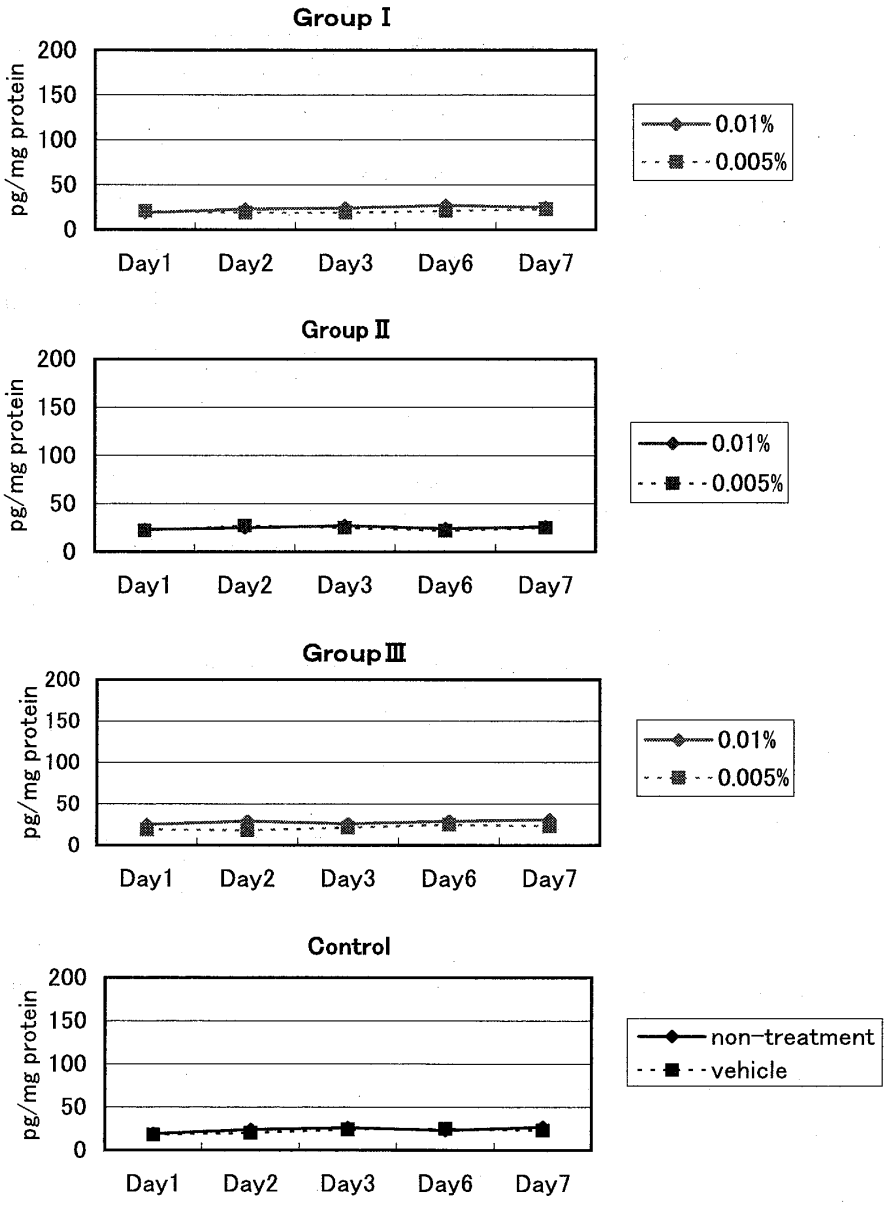

Fig.4. Content by ELISA expression level of IL-2 after treatment

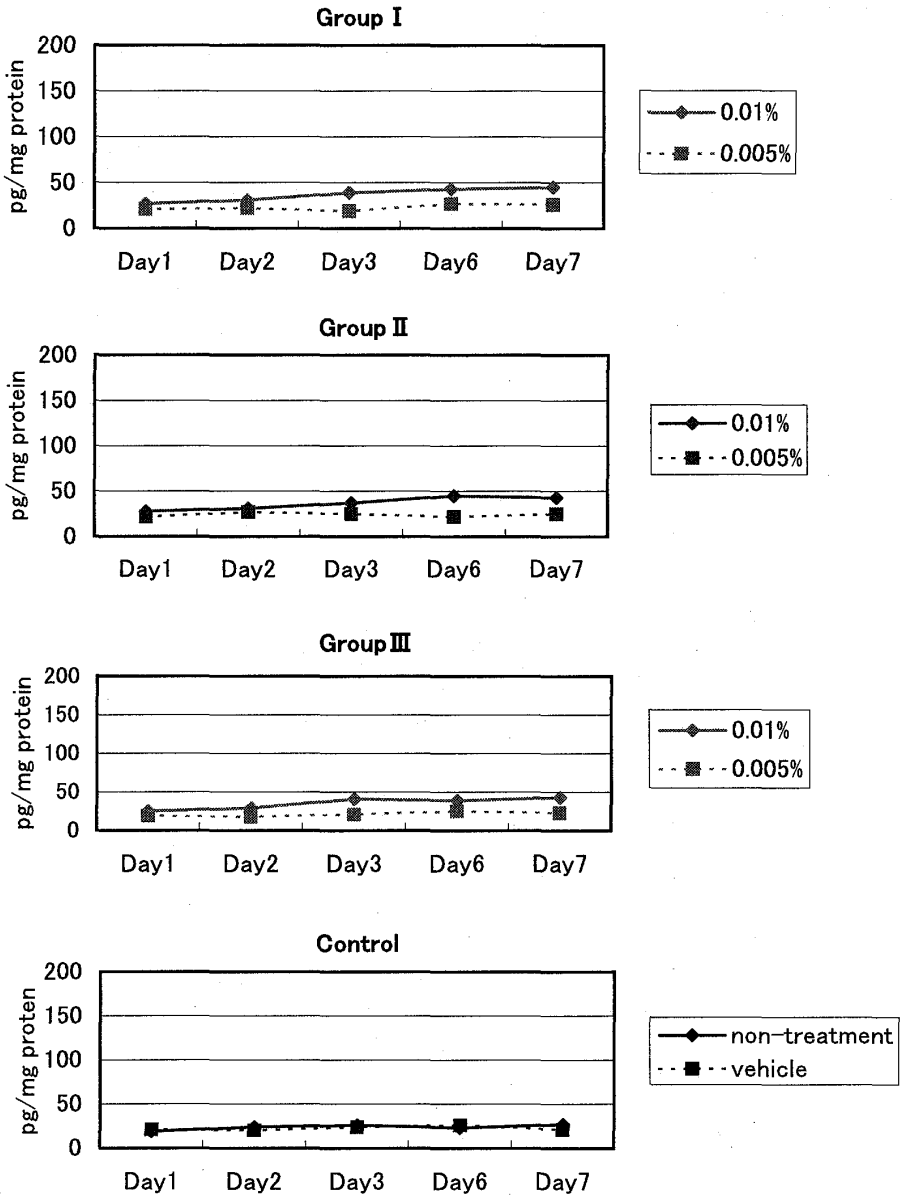

Fig.5. Content by ELISA expression level of IFN-γ after treatment

4. Discussion

CPS is a cutaneous allergic reaction induced by skin contact with allergenic chemicals and exposure to ultraviolet light at specific wavelengths. CD4-positive T cells are involved in inducing CPS as well as contact sensitivity (CS). [1-3] In CPS, which is a delayed-type hypersensitivity mediated by CD4-positive T cells, a suppressing pathway exists as in other delayed-types of hypersensitivity. [5,6] CD4-positive T cells are also involved in the suppressing pathway, where helper T cells (Th) are restricted by class II molecules, I-A, on APC, while suppressor T cells (Ts) are restricted by I-E. [7] Mouse CD4-positive T cells are classified into Th1 and Th2 according to the cytokine profile .[10] It is becoming clear that Th and Ts are Th1 and Th2, respectively, suggesting that the ratio of Th1 and Th2 determines the degree of delayed-type hypersensitivity.

We have been analyzing the patterns of cytokine expression in local skin reactions using mouse delayed-type hypersensitivity models. The cytokines

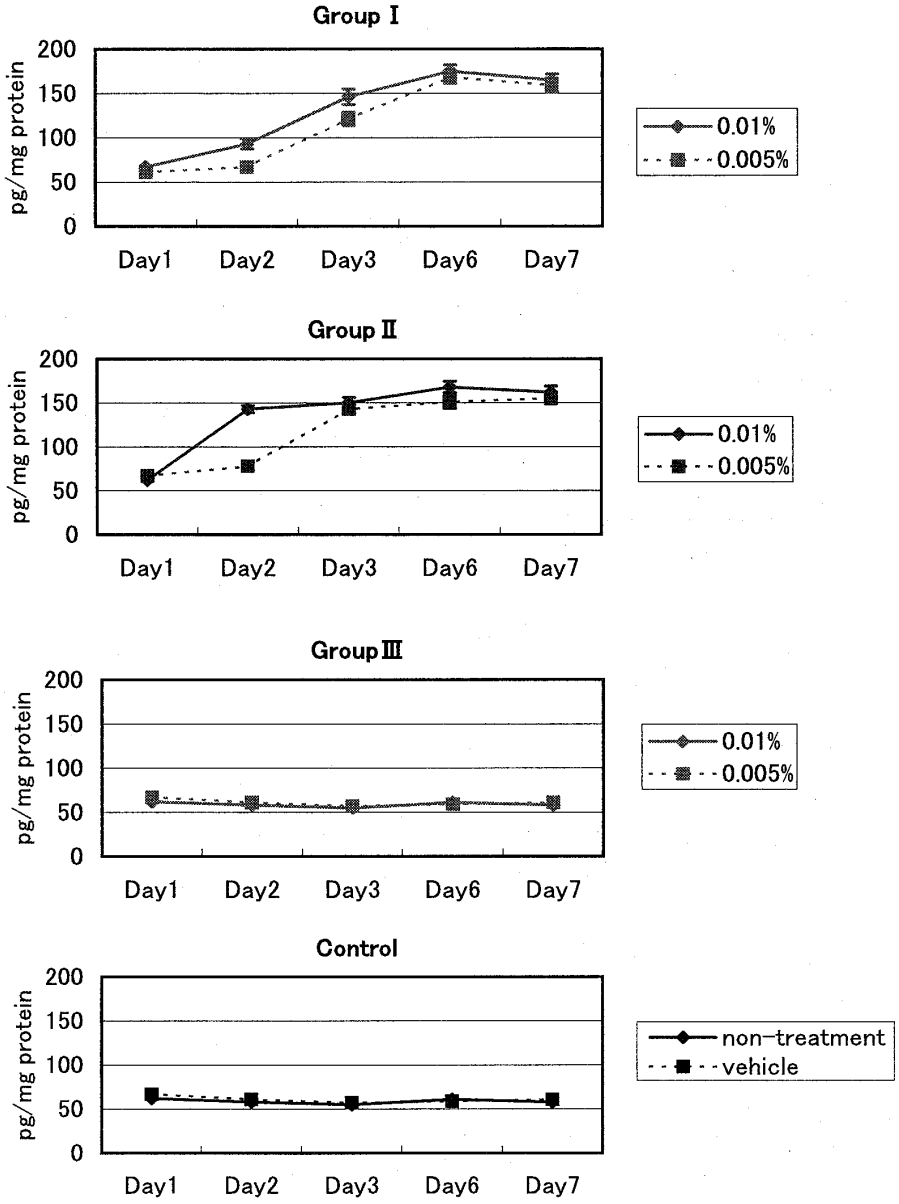

Fig.6. Content by ELISA expression level of IL-4 after treatment

expressed in CS induced by DNFB were predominantly Th1 cytokines such as IL-2 and IFN-γ [11-14], while in CPS induced by TCSA, expression of Th1 cytokines such as IL-2 and IFN-γ was lower than in CS, and early expression of Th2 cytokines such as IL-4 and IL-10 was observed. [15] To investigate the Th1/Th2 ratio in CPS, we performed similar experiments using mice (H-2^k) in which Th2 cytokines are easily induced due to differences in the H-2 haplotype, and found that the H-2^k haplotype mice showed low CPS responses, and Th2 cytokines such as IL-4 were strongly expressed. This suggests that Th2 cytokines were closely involved in the immunological pathway, and that CPS is the reaction system in which Th2 cytokines are easily induced. [9]

In the present study, we attempted to develop an in vitro screening system for photo allergens using spleen cells in photosensitized mice. To confirm the cellular immunity in mice with CPS induced by TCSA, we conducted the following experiments. Spleen cells in

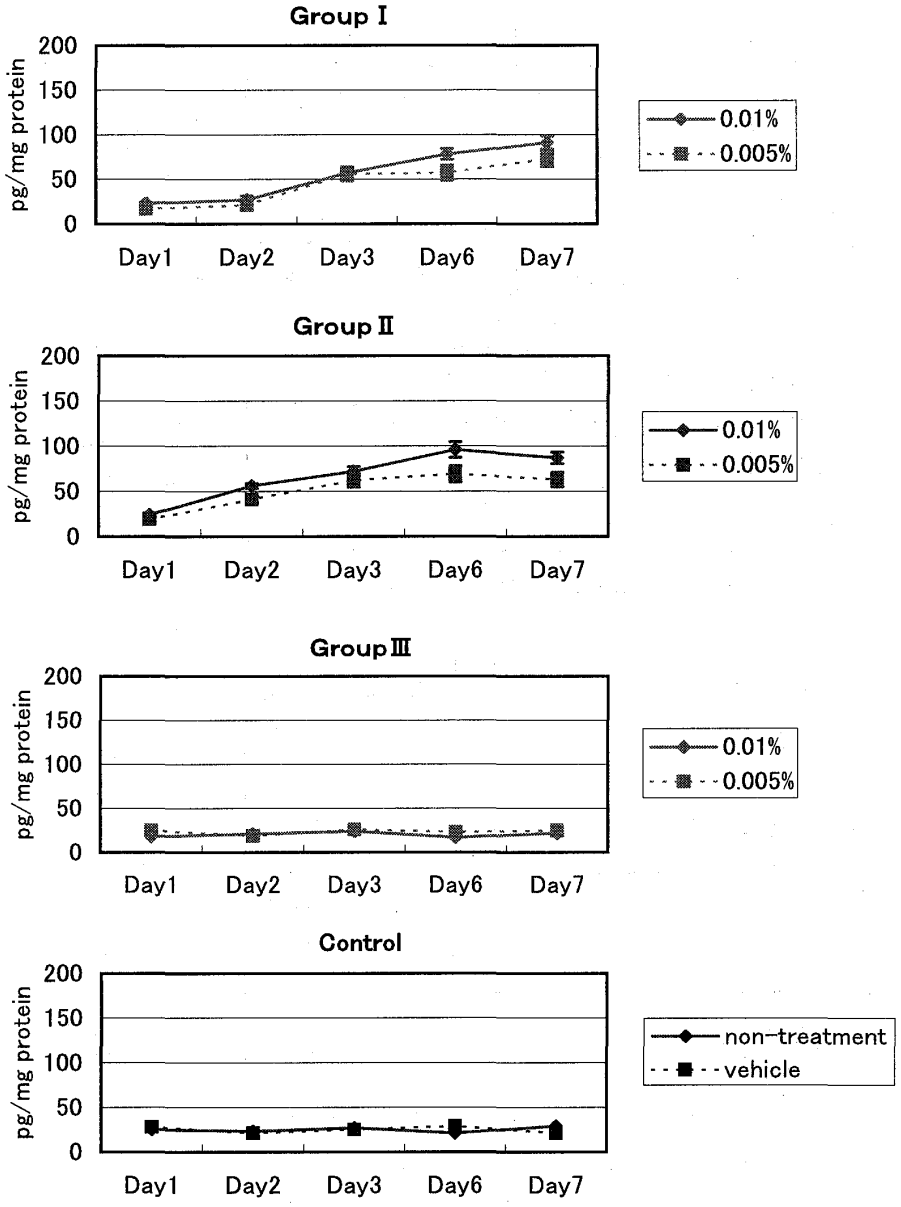

Fig.7. Content by ELISA expression level of IL-5 after treatment

mice with CPS induced by TCSA were introduced into untreated mice, and CPS reaction was induced by introducing TCSA into the mouse auricle 24h after spleen cell introduction. The auricle swelling, in which the peak was 24-48h after induction, was almost the same level as in CPS induced by TCSA. This result confirmed that cell transfer was possible in the CPS system induced by TCSA as in CS induced by DNFB.

Next, cytokine mRNA expressed in skin reaction regions in mice with CPS induced by TCSA was analyzed. The expression of cytokines was also analyzed and compared in mice with CPS induced by ordinary TCSA and UVA-irradiated TCSA. TCSA was applied to the auricle of mice photosensitized by TCSA, and the mice were divided into 2 groups, of which one received UVA irradiation after TCSA application to the auricle (group I, ordinary photosensitization group) and the other received application of UVA-irradiated TCSA to the auricle (group II). In both groups, ear swelling and the expression of cytokines in the auricle were examined by RT-PCR 24 h after induction of CPS reaction. A clear peak of ear swelling was observed 24-48h after

induction in both groups. A small amount of Th1 cytokines such as IFN-γ was expressed, and Th2 cytokines such as IL-4 were observed in large amounts in both groups. In the group treated by UVA irradiation alone after photosensitization (group III) and the group treated by TCSA application alone after photosensitization (group IV) prepared as the negative control groups, ear swelling was barely observed, but low levels of expression of Th1 and Th2 cytokines were observed. These results suggested that the same reaction as in ordinary CPS was induced by UVA-irradiated TCSA. It has been reported that marked expression of Th2 cytokines such as IL-5 was observed in a similar experimental system using humans after induction using UVA-irradiated photo allergens other than TCSA. [16] Therefore, it was considered that application of a specific UVA-irradiated photo allergen to photosensitized animals (mice) generated photomodified epidermal cells by the same photo allergens as in ordinary CPS, and that a CPS-like reaction was observed by processing with APC. By subtracting the expression of Th1 and Th2 cytokines in the negative control groups III and IV from that in groups I and II, respectively, marked expression of Th2 cytokine IL-4 was evident, suggesting that IL-4 was the key cytokine in generating CPS reaction.

The induction of in vitro CPS reaction in total spleen cells of mice photosensitized by TCSA was examined by analyzing cytokine expression as the parameters. Experiments were performed using 3 groups; group I with spleen cells treated by TCSA addition and then UVA irradiation (ordinary photosensitization group), group II with spleen cells treated by addition of UVA-irradiated TCSA, and group III with spleen cells treated by UVA irradiation and then TCSA addition. The expressions of cytokines in the supernatants of culture media in the 3 groups were examined by ELISA. In groups I and II, the expression of Th2 cytokines, IL-4 and IL-5, gradually increased, of which the patterns were similar in both groups. These results agreed with those obtained by in vivo examination, suggesting that UVA-irradiated TCSA induced reactions equivalent to CPS in the in vitro system as in the in vivo system. In the in vitro system, Th2 cells were predominant compared to the in vivo system, indicating that the involvement of Th1 cells was very weak, but the involvement of Th2 cells was strong at the level of lymphocytes. Since cytokines are produced in skin reaction regions not only by lymphocytes but also by keratinocytes and macrophages, the expression of various cytokines was observed, while in the spleen cells (lymphocytes), Th2 lymphocytes, which are more specialized in the CPS immune system, were considered to be markedly expressed. Our results support the importance of Th2 cytokine expression for CPS reaction indicated in past studies. [9, 15] In the present study, we used spleen cells, but experiments using serum will also be possible. Since experiments using serum can be performed without killing animals, such experimental models are desirable from the viewpoint of animal welfare. Furthermore, experimental models using serum can be used for the examination of human light hypersensitivity induced by photo allergens. Although more detailed studies are necessary, this study suggested that in vitro screening of photosensitization allergens is possible to some extent by analysis of Th2 cytokine expression.

References

1) Takigawa M., Miyachi Y., *J. Invest. Dermatol.*, **79**, 108-115 (1982).

2) Miyachi Y., Takigawa M., *Arch. Dermatol.*, **119**, 736-739 (1983).

3) Tokura Y., Takigawa M., Eur. J. Dermatol., **3**, 87-91 (1993).

4) Maguire H. C., Kaidbey K., *J. Invest. Dermatol.*, **79**, 147-152 (1982).

5) Tokura Y., Satoh T., Takigawa M., Yamada M., *J. Invest. Dermatol.*, **94**, 471-476 (1990).

6) Miyachi Y., Takigawa M., J. Invest. Dermatol., **78**,

363-365 (1982).
7) Tokura Y., Yagi H., Satoh T., Takigawa M., *J. Invest. Dermatol.*, **101**, 673-678 (1993).
8) Tokura Y., Satoh T., Yamada M., Takigawa M., *Cell Immunol.*, **135**, 195-207 (1991).
9) Suzuki K., Yamazaki S., Tokura Y., *J. Dermatol. Science*, **144**, 138-144 (2000).
10) Mosmann T. R., Coffman R. L., *Ann. Rev. Immunol.*, **7**, 145-173 (1989).
11) Yamazaki S., Kuwahara H., Kakishima H., *Yakugaku Zasshi.*, **117**, 155-161 (1997).
12) Yamazaki S., Kuwahara H., Kakishima H., *Yakugaku Zasshi.*, **117**, 162-166 (1997).
13) Yamazaki S., Suzuki K., Kakishima H., *Environ. Dermatol.*, **4**, 177-183 (1998).
14) Suzuki K., Yamazaki S., Kakishima H., *Environ. Dermatol.*, **5**, 93-100 (1998).
15) Yamazaki S., Miyake R., Suzuki K., Kakishima H., *Jpn. J. Dermatol.*, **108**, 9-14 (1998).
16) Miyata T., Fujimura T., Asai T., Katsuoka K., Japanese Society for Immunology, **26**, 200 (1996).

Figure legends

Fig.1. Ear swelling responses of CPS to TCSA in Balb/c mice.
*$P<0.05$ compared to control (regular photosensitive group), Student's t-test

Fig.2. Ear swelling responses of CPS to TCSA in Balb/c mice.
Group Ⅰ; regular photosensitivity group
Group Ⅱ; applying UVA irradiated photo hapten (TCSA)
Group Ⅲ; UVA irradiated, no application of TCSA
Group Ⅳ; TCSA application, no UVA irradiation
*$P<0.05$ compared to control (regular photosensitive group), Student's t-test.
**$P<0.01$ compared to control (regular photosensitive group), Student's t-test

Fig.3. RT-PCR analysis of cytokine mRNA expression in the ear after treatment.
Group Ⅰ; regular photosensitivity group
Group Ⅱ; applying UVA irradiated photo hapten (TCSA)
Group Ⅲ; UVA irradiated, no application of TCSA
Group Ⅳ; TCSA application, no UVA irradiation
Lane1; 0h,lane 2;12h, lane 3;24h, lane 4;48h, lane 5;72h, bp;base pairs

Fig.4. Contact by ELISA expression level of IL-2 after treatment.
Group Ⅰ; photohapten (TCSA) was added to the cells and then UVA irradiation is performed (regular photosensitivity group)
Group Ⅱ; UVA-treated photo hapten (TCSA) was added to the cells
Group Ⅲ; photo hapten (TCSA) was added to the UVA-treated cells

Fig.5. Contact by ELISA expression level of IFN-γ after treatment.
Group Ⅰ; photohapten (TCSA) was added to the cells and then UVA irradiation is performed (regular photosensitivity group)
Group Ⅱ; UVA-treated photo hapten (TCSA) was added to the cells
Group Ⅲ; photo hapten (TCSA) was added to the UVA-treated cells

Fig.6. Contact by ELISA expression level of IL-4 after treatment.
Group Ⅰ; photohapten (TCSA) was added to the cells and then UVA irradiation is performed (regular photosensitivity group)
Group Ⅱ; UVA-treated photo hapten (TCSA) was added to the cells
Group Ⅲ; photo hapten (TCSA) was added to the UVA-treated cells

Fig.7. Contact by ELISA expression level of IL-5 after treatment.
Group Ⅰ; photohapten (TCSA) was added to the cells and then UVA irradiation is performed (regular photosensitivity group)

Group Ⅱ; UVA-treated photo hapten (TCSA) was added to the cells

Group Ⅲ; photo hapten (TCSA) was added to the UVA-treated cells

要旨

3, 3', 4', 5- tetrachlorosalicylanilide (TCSA) によって光接触過敏症（contact photosensitivity；CPS）を誘発したマウスの脾細胞を、未処置マウスに細胞移入した。細胞移入24時間後に、マウスの耳介部にTCSAでCPS反応を惹起した結果、通常のTCSAによるCPSと同レベルの耳介腫脹反応が認められた。次に、TCSAにより光感作誘導処置を行ったマウスを用いて、UVA照射済み光ハプテン（TCSA）塗布および、TCSAを塗布した後にUVA照射することでCPS反応を惹起し、24時間後の耳介腫脹および反応部位（耳介部）に発現するサイトカインをRT-PCR法で解析を行った。その結果、両群とも耳介腫脹は顕著に認められ、それに加えてIL-4mRNA発現も同程度に認められた。これらの結果から、TCSAによって誘導されるCPSは細胞移入により誘発が可能な系であること、並びに、あらかじめUVA照射したTCSAで惹起できることが明らかになった。そこで次に、TCSAによる光感作誘発マウスの脾細胞を用いてCPS反応を、in vitroにおいてTh2型サイトカインの産生を指標に検討を行った。光ハプテン（TCSA）を添加後UVA照射を施す群（Ⅰ群：いわゆる、通常の光感作群）、UVA照射済み光ハプテン（TCSA）を細胞に添加する群（Ⅱ群）および細胞にUVA照射を施した後、光ハプテン（TCSA）を添加する群（Ⅲ群）の3群構成で、各々の群の培養上清中のサイトカイン量をELISAにて測定した。その結果、特にⅠ群とⅡ群においてIL-4及びIL-5の産生量が上昇傾向にあり、Th2型サイトカインがCPSの反応成立の際に重要であるという過去の実験データと一致した。以上の結果から、Th2型サイトカインの産生を指標にして、光感作アレルギー物質（光アレルゲン）のin vitroによるスクリーニングがある程度可能であることが示された。

（2006.10.23 受稿）