

were also the major antigens for specific IgE production even in the non-atopic children. It is also very interesting that both the total and specific IgE levels significantly increased with age in children without AD. The tendency of age-related accumulation of total and specific IgE was not observed in the children with AD, because it had already reached high levels as early as at age 1. Nolles et al. (27) reported a similar age-related increase of IgE antibodies. These results suggest (1) that the total and specific IgE levels increase with age probably with cumulative exposure to common allergens even in non-atopic individuals, and (2) that earlier and higher increases in total and specific IgE antibodies are associated with AD.

The prevalence of AD has been studied in a variety of populations throughout the world (6-13), but comparisons of prevalence is difficult because of differences in study populations and study methods. Some investigators have measured point prevalence (28, 29), while others have measured 12 months prevalence in different age groups (8). The variation problem of study designs was addressed by the International Study for Asthma and Allergies in Childhood (ISAAC) (8). The methodology was subsequently standardized by the use of a questionnaire based on the U.K. Working Party definition of AD (17). A previous validity study suggested that the questionnaire might slightly overestimate the true prevalence (30). In the present study, we translated the questionnaire of the U.K. Working Party into Japanese and analyzed the sensitivity and specificity of the translated questionnaire. Although the specificity of the translated questionnaire for AD was 94.7%, its sensitivity was only 59 %. This low sensitivity may be due to some incomprehensibility in the Japanese translation and to insufficient parent cooperation. It is critical that we refine the translation to improve the parents' understanding of the translated questionnaire.

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FIGURE LEGENDS

Figure 1

Levels of total IgE in children 5 years of age and younger in nursery schools, Ishigaki City, Okinawa, Japan, in 2001.

The black bar (■) indicates children with AD and the open bar (□) shows children without AD. The standard deviations are shown by the thin, vertical bars and statistical significance is indicated by the “*” ($P < 0.05$) and “***” ($P < 0.001$).

Fig.1

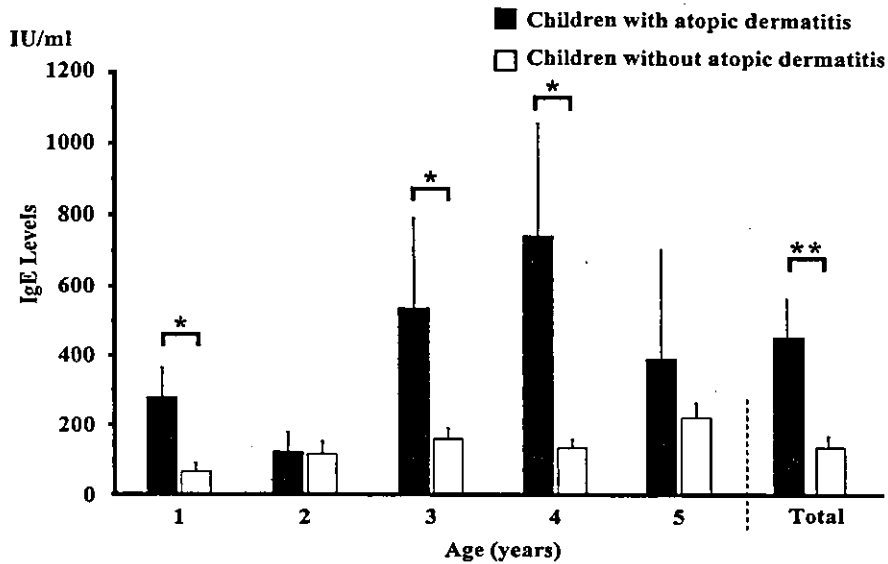


Table 1 The prevalence of atopic dermatitis (AD) in children aged 5 years and under in nursery schools in Ishigaki City, Okinawa, Japan, in 2001

Age (years)	Boys		Girls		Total	
	Numbers examined	AD Cases (%)	Numbers examined	AD Cases (%)	Numbers examined	AD Cases (%)
1	47	2 (4.3)	41	4 (9.8)	88	6 (6.8)
2	57	3 (5.3)	56	3 (5.4)	113	6 (5.3)
3	78	5 (6.4)	52	8 (15.4)	130	13 (10.0)
4	65	5 (7.7)	68	4 (5.9)	133	9 (6.8)
5	55	4 (7.3)	46	1 (2.2)	101	5 (5.0)
Total	302	19 (6.3)	263	20 (7.6)	565	39 (6.9)

Table 2 Comparison of the rates of abnormal total IgE levels between children with and without atopic dermatitis (AD) in Ishigaki City, Okinawa, Japan, in 2001

Age (years)	With AD		Without AD	
	No. tested	Abnormal IgE No. # (%)	No. tested	Abnormal IgE No. # (%)
1	6	3 (50.0)	82	3 (3.7)**
2	6	1 (16.7)	107	7 (6.5)**
3	13	3 (23.1)	117	23 (19.7)**
4	9	5 (55.6)	124	15 (12.1)**
5	5	1 (20.0)	96	19 (19.8)**
Total	39	13 (33.3)*	526	67 (12.7)

A total IgE level of over 230 IU/ml was considered abnormal.

* A statistically significant difference was found between children with and without atopic dermatitis (P=0.0029)

** P=0.0007, calculated by use of the Cochran Armitage test.

Table 3 Comparison of positive specific IgE antibody responses of children in Ishigaki City, Okinawa, Japan, in 2001, with and without atopic dermatitis (AD)

Specific IgE antibody	AD children (n=39)		Non-AD children (n=526)		P value *
	Positive No.	(%)	Positive No.	(%)	
house dust	17	(43.6)	115	(21.9)	0.0038
Japanese cedar pollen	0	—	0	—	—
<i>D. pteronyssinus</i>	19	(48.7)	122	(23.2)	0.0008
<i>D. farinae</i>	19	(48.7)	98	(18.6)	< 0.0001
<i>Candida</i>	2	(5.3)	2	(0.4)	0.0255
<i>Malassezia</i>	0	—	1	(0.2)	—
chicken egg white	10	(25.6)	57	(10.8)	0.0167
cow's milk	9	(23.1)	68	(12.9)	0.1233
rice	1	(2.6)	0	—	—
soy	2	(5.3)	2	(0.4)	0.0255
one or more antibodies	25	(64.1)	159	(30.2)	< 0.0001

* P values represents the result of statistical comparison of children with and without atopic dermatitis.
D. pteronyssinus, *Dermatophagoides pteronyssinus*; *D. farinae*, *Dermatophagoides farinae*

Table 4 Comparison of positive rates for one or more specific IgE antibodies in children with and without atopic dermatitis (AD) in Ishigaki City, Okinawa, Japan, in 2001 by age

Age (years)	with AD		without AD	
	No. tested	Abnormal No. (%)	No. tested	Abnormal No. (%)
1	6	3 (50.0)	82	21 (27.6)**
2	6	5 (83.3)	107	29 (27.1)**
3	13	5 (38.5)	117	36 (30.8)**
4	9	9 (100.0)	124	32 (25.8)**
5	5	3 (60.0)	96	41 (42.7)**
Total	39	25 (64.1) *	526	159 (30.2)

* A statistically significant difference was found between children with and without atopic dermatitis (P<0.0001)

** P=0.0394, calculated by use of the Cochran Armitage test.

Table 5

Responses to the United Kingdom Working Party questionnaire for children with and without AD diagnosed by clinical examination

Questionnaire	Physical examination	
	AD N=39	Non-AD N=526
AD N=51	23 (59%)	28 (5.3%)
Non-AD N=514	16 (41%)	498 (94.7%)

Ubiquitin-fusion degradation pathway plays an indispensable role in naked DNA vaccination with a chimeric gene encoding a syngeneic cytotoxic T lymphocyte epitope of melanocyte and green fluorescent protein

MANXIN ZHANG,* KAZUNARI ISHII,* HAJIME HISAEDA,* SHIGEO MURATA,† TOMOKI CHIBA,‡ KEIJI TANAKA,‡ YANG LI,* CHIKAGE OBATA,† MASUTAKA FURUE† & KUNISUKE HIMENO* *Departments of *Microbiology and Immunology, †Dermatology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan and ‡Department of Molecular Oncology, The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan*

SUMMARY

Antitumour immunity against murine melanoma B16 was achieved by genetic immunization with a naked chimeric DNA encoding a fusion protein linking green fluorescent protein (GFP) to the N-terminus of a major CD8⁺ cytotoxic T lymphocyte (CTL) epitope of tyrosinase-related protein 2 (TRP-2_{181–188}) of murine melanoma, designated as pGFP-TRP-2. Tumour growth was profoundly suppressed in C57BL/6 mice immunized with pGFP-TRP-2, while mice vaccinated with pTRP-2 showed rapid tumour growth and died within 40 days after tumour challenge. Splenocytes of mice immunized with pGFP-TRP-2 showed high CTL activity specific for TRP-2_{181–188}. GFP-TRP-2 expressed in COS-7 cells was rapidly degraded *in vitro* and the degradation was almost completely prevented by adding a proteasome inhibitor, MG-132, in the culture. Furthermore, the antimelanoma immunity induced by genetic immunization with pGFP-TRP-2 was completely cancelled in mice deficient in proteasome activator PA28 α/β . Taken together, GFP-TRP-2 processed by cytosolic proteasome played a central role in breaking peripheral tolerance to a melanoma/melanocyte antigen, TRP-2_{181–188}, by activating CD8⁺ CTL specific for TRP-2_{181–188}. TRP-2_{181–188} fused to GFP may be readily cut off from GFP by the ubiquitin-fusion degradation (UFD) pathway and efficiently presented to major histocompatibility complex class I molecules, resulting in effective induction of CD8⁺ T cells specific for the CTL epitope. Furthermore, CD4⁺ T cells specific for GFP were shown to play a crucial role in the antimelanoma immunity, probably potentiating activity of TRP-2-specific CTL and/or the 'ubiquitin-proteasome pathway'. It is noteworthy to document that genetic immunization with pGFP plus pTRP-2_{181–188} failed to exert the antitumour immunity.

Keywords Melanoma, UFD pathway, TRP-2, DNA vaccine, GFP

INTRODUCTION

Antigen presentation to CD8⁺ T cells is mediated by major histocompatibility complex (MHC) class I molecules expressed on antigen-presenting cells (APC)/

dendritic cells (DC). Primarily, CD8⁺ T cells recognize MHC class I-associated peptides derived from endogenous antigens, such as oncogene-products or viral antigens, located in the cytosol. Prior to antigen presentation by MHC class I molecules, antigens must be ubiquitinated and then be processed into antigenic peptides by the proteasome. This antigen processing system is termed as 'ubiquitin-proteasome pathway'.^{1–3} Vaccination with naked DNA encoding endogenous antigens is an efficient means for induction of antigen-specific immunity, particularly activation of CD8⁺ T cells, as those intracellularly expressed gene product, antigenic protein, must be exclusively processed through the ubiquitin-proteasome

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Correspondence: Dr K. Himeno, Department of Microbiology and Immunology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku Fukuoka 812-8582, Japan. E-mail: himeno@parasite.med.kyushu-u.ac.jp

pathway and the created MHC class I-binding epitopes activate specific CD8⁺ T cells.

A large number of melanoma antigens able to activate immune responses have been identified.⁴⁻⁶ Among them, melanocyte lineage differentiation antigens (MDAs) are the most prominent in induction of immune responses. The tyrosinase-related protein 2 (TRP-2) is one of MDAs expressed by normal and malignant melanocytes both in humans and mice.^{7,8} Several epitopes recognized by human CD8⁺ T cells have been identified on the TRP-2 protein.⁹ Bloom *et al.* identified a major cytotoxic T lymphocyte (CTL) epitope, TRP-2₁₈₁₋₁₈₈, recognized within the H-2K^b MHC class I molecules, and they reported that passive transfer of TRP-2₁₈₁₋₁₈₈-specific CTLs into C57BL/6 (B6) mice is therapeutic against established B16 lung metastasis.⁸ Naive B6 mice have significant numbers of CTL precursors with reactivity to TRP-2₁₈₁₋₁₈₈. However, the immune response to TRP-2₁₈₁₋₁₈₈ may be limited by the tolerance mechanisms that only allow low-affinity lymphocytes to survive after T-cell selection in the thymus.¹⁰ Overcoming these tolerizing limitations may be critical to the development of clinical immunotherapeutic approaches for patients with melanoma. Self-tolerance is broken down in certain conditions. One case is that self-antigens are effectively presented to MHC class I molecules on professional APC/DC, then those tolerant CD8⁺ T cells are activated.¹¹ Another case is that CD4⁺ T cells recognizing MHC class II-associated peptides of target antigens support to break the tolerance of CD8⁺ T cells specific for autologous tumour antigens via releasing some cytokines such as interleukin-12 (IL-12), probably through activation of DC.¹²

Previous experiments demonstrated that genetic immunization of B6 mice with cDNA encoding autologous murine TRP-2 as a model antigen was unable to induce significant protective immunity against B16 melanoma cells naturally expressing TRP-2.¹³ We supposed that failure in breaking peripheral tolerance of CD8⁺ T cells by ordinary DNA vaccinations is explained in two ways. The first cause may be that tag-molecules which lead antigenic peptide to the ubiquitin-proteasome pathway are not contained or insufficient in some antigenic proteins. The second cause should be that MHC class II-binding epitopes are not contained in the TRP-2, especially TRP-2₁₈₁₋₁₈₈. Green fluorescent protein (GFP) is known to be readily targeted by ubiquitin fusion degradation (UFD) pathway, a virtual route of the ubiquitin-proteasome pathway.¹⁴

In this study, we investigated the effect of DNA vaccination with a gene encoding TRP-2₁₈₁₋₁₈₈ fused to GFP (pGFP-TRP-2) on murine melanoma tumour growth *in vivo* using B16 cells, a spontaneous and poorly immunogenic murine melanoma cell line. Strikingly, immunization with pGFP-TRP-2 induced potent protective immunity against the tumour. It is noteworthy that immunization neither with pTRP-2₁₈₁₋₁₈₈ alone nor with pGFP plus pTRP-2₁₈₁₋₁₈₈ induced the antitumour immunity. A key role of the ubiquitin-proteasome pathway in the present study was confirmed *in vitro* using a proteasome inhibitor, MG-132 and *in vivo* using proteasome activator PA28 α/β -deficient mice.

MATERIALS AND METHODS

Animals and tumours

We performed mouse studies in accordance with the institutional guidelines of Kyushu University. Female C57BL/6 (B6) mice between 7 and 8 weeks of age were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Proteasome activator PA28-knockout (PA28 α/β ^{-/-}) mice of B6 background were also used.¹⁵ B16F1 and B16F10, murine melanoma cell lines, were maintained *in vitro* in RPMI-1640 supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μ g/streptomycin, 20 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulphonic acid, 50 mM NaHCO₃ and 2 mM L-glutamine.

Plasmids

pGFP plasmid, purchased from Clontech (Palo Alto, CA), was used to construct GFP-TRP-2 plasmid: GFP-TRP-2₁₈₁₋₁₈₈ DNA was amplified by PCR using sense 5'-CAG AGAATTCAGACGCCACCATGGTG-3' and antisense 5'-CCATGGATCCTATTAGAGCCACACAAAAAAGT CATAACCTTGTACAGCTCGTC-3' primers and using pGFP plasmid DNA as a template. The PCR product of GFP-TRP-2₁₈₁₋₁₈₈ DNA was inserted into *Eco*RI and *Bam*HI sites of a pcDNA 3.1 (-) vector (Invitrogen, San Diego, CA). A pTRP-2 plasmid encoding TRP-2₁₈₁₋₁₈₈ was constructed as follows: DNA was amplified by polymerase chain reaction (PCR) using sense 5'-CAGAGAATTC AAGGCATGGTGTATGACTTTTTTGTGTGGCTCTA ATAG-3' and antisense 5'-ACGTCTTAAGAGATATCCA GCACAGT-3' as primers and using pGFP-TRP-2 plasmid DNA as a template. After treatment with *Dpn*I, the PCR product was ligated and used for transformation of *Escherichia coli*.

In vivo gene transfer and implantation of melanoma cells

We used a Helios Gene Gun (Bio-Rad, New York, NY) as previously described.^{16,17} Briefly, plasmid DNA was precipitated onto 1.6 μ g gold particles and coated on the inner surface of tubing by a tube loader. The final tubing segment enabled delivery of 0.125 mg gold particles and 2 μ g plasmid DNA per transfection. pTRP-2, pGFP-TRP-2 or pGFP were transferred to three different portions of shaved abdominal skin three times at 1-week intervals. A total of 18 μ g plasmid was administered to each mouse. One week after the last vaccination, 2 \times 10⁵ melanoma cells in 0.1 ml of phosphate-buffered saline (PBS) were injected subcutaneously (s.c.) into each B6 mouse. Tumours were measured twice a week using a caliper, and tumour volume was calculated as $\pi/6 \times [(a \times b)^{1/2}]^3$, where *a* and *b* are two perpendicular major diameters.

In vitro transfection and Western blotting

Five hundred thousand COS-7 cells in a 2.5-cm dish (Nunc, Roskilde, Denmark) were transfected with 2 μ g of each plasmid DNA by using Lipofectamine. Twenty-four hr after transfection, cell lysates were prepared by adding 200 μ l lysate buffer (50 mM Tris-HCl/1% Nonidet P-40/1% sodium dodecyl sulphate (SDS)/1 μ M

leupeptin/100 μM phenylmethylsulphonylfluoride/1 μM pepstatin A/100 μM ethylenediamine tetraacetic acid), and 10 μg protein was used for Western blotting with anti-HA (mouse monoclonal antibody clone 12CA5, Roche, Mannheim, Germany) diluted at 1 : 2000 as the first antibody. Peroxidase-conjugated anti-mouse immunoglobulin G (IgG) (H + L) (Zymed, San Francisco, CA) was the second antibody. Binding antibodies were detected by using enhanced chemiluminescence (ECL) reagents (Amersham, Amersham, UK). Anti-GFP monoclonal antibody JL8 (Clontech) diluted at 1 : 1000 was used as the first antibody for detecting GFP and GFP-TRP-2 expression. Proteasome inhibitor MG-132 (Bostonchem, Boston, MA) was added at 10 $\mu\text{g}/\text{ml}$ 24 hr after transfection, and cell lysates were prepared 24 hr after addition of MG-132.

Cytotoxicity assay

The H-2K^b-binding peptide VYDFFVWL (TRP-2₁₈₁₋₁₈₈) was synthesized by an Fmoc/PyBOP strategy and purified by high-pressure liquid chromatography (SAWADY Technology, Tokyo, Japan). Peptides were dissolved at 10 $\mu\text{g}/\text{ml}$ in PBS containing 10% dimethylsulphoxide and stored at -20° . Mice were killed at the time of tumour challenge, and their spleen cells (5×10^6) were cocultured with 10^5 mitomycin C-treated B16F1 cells in 24-well culture plates in complete RPMI-1640 media. After culturing for 5 days *in vitro*, graded numbers of viable effector cells and [³H]thymidine-labeled EL-4 cells (10^4), pulsed for 2 hr with TRP-2₁₈₁₋₁₈₈ peptide (4 $\mu\text{g}/\text{ml}$), were placed into the round-bottomed wells of 96-well plates. After 4.5 hr of incubation, the cells and their media were harvested onto glass-fibre filters, and then radioactivity level was assessed using a beta scintillation counter and specific killing was calculated.

In vivo depletion of T-cell subsets

To determine the relative contributions of T cells to anti-tumour immunity, antibodies corresponding to each T-cell subset were administered to mice. Anti-CD4 monoclonal antibody (mAb; clone GK1.5) or anti-CD8 mAb (clone 53-6.72) was injected intraperitoneally at 0.5 mg per injection per mouse on days -3, -1, 1 and 3. Tumour challenge was on day 0. Depletion of each T-cell subset was confirmed by flow cytometric analysis, and over 95% of the cells of the appropriate subsets were regularly found to be depleted.

Enzyme-linked immunosorbent assay (ELISA)

A 96-well plate (Nunc) was coated with 100 μl of GFP (Clontech) at 1.0 $\mu\text{g}/\text{ml}$ in PBS overnight at 4° . To prevent non-specific binding, 200 μl of blocking buffer (0.1% bovine serum albumin, 0.05% Tween-20 in PBS, pH 7.2) was added to a coated plate, and the plate was incubated at room temperature for 1 hr. Then serum samples (each 100 μl) from pGFP-TRP-2-immunized mice and control mice, diluted 1000–27 000-fold with PBST (0.05% Tween-20 in PBS), were placed in duplicate wells. After 1.5 hr of incubation, the plate was washed four times with PBST and then incubated with 100 μl of the second antibody (anti-mouse IgG-horseradish peroxidase, Pierce, Rockford, IL)

diluted 1000-fold with PBST for 1.5 hr. After washing five times, bound antibodies were visualized by the addition of a substrate of horseradish peroxidase (OPD, Sigma, St Louis, MO). Intensity was measured as OD at 450 nm by using an ELISA reader (Labsystem Multiskan, Finland).

RESULTS

Construction of plasmids encoding TRP-2₁₈₁₋₁₈₈ or GFP-TRP-2₁₈₁₋₁₈₈

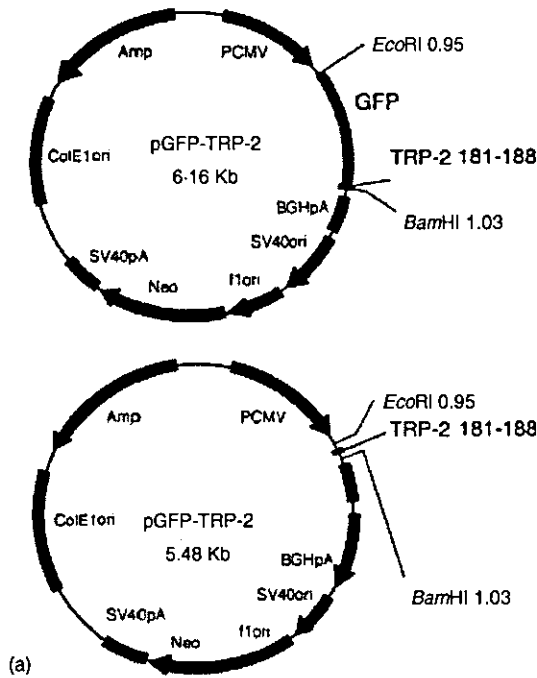
A GFP-TRP-2₁₈₁₋₁₈₈ expression plasmid was constructed by PCR using pGFP as a template and the primers shown in Materials and Methods (pGFP-TRP-2), and a TRP-2₁₈₁₋₁₈₈ expression plasmid was constructed by PCR using pGFP-TRP-2 as a template and the primers shown in Materials and Methods (pTRP-2). Construction of pTRP-2 and pGFP-TRP-2 were confirmed by DNA sequencing. The expression of fusion GFP-TRP-2 was confirmed by Western blotting after transfection of COS-7 cells with these plasmids (Fig. 1). A single band was detected in cells transfected with pGFP or pGFP-TRP-2. GFP alone was strongly expressed more than the fusion GFP-TRP-2.

Antitumour effect of gene gun-mediated vaccination with fusion pGFP-TRP-2

To determine the antitumour effect of gene gun-mediated vaccination, we delivered pTRP-2, pGFP plus pTRP-2 or pGFP-TRP-2 into the abdominal skin of B6 mice before challenge with syngeneic B16F1 melanoma cells. Rapid tumour growth was seen in mice vaccinated with control pGFP, and the mice died usually 30–40 days after the challenge, findings similar to those in a group of the mice that had not been vaccinated. Vaccination with pTRP-2 or pGFP plus pTRP-2 did not influence tumour growth and could not rescue mice. Mice vaccinated with pGFP-TRP-2 showed limited tumour growth, a part of which survived with a trace of tumour growth, although some mice gradually died much later than control mice (Fig. 2). Those mice that had rejected B16F1 cells following pGFP-TRP-2 vaccination readily acquired resistance to a challenge even with much higher tumorigenic B16F10 (data not shown), indicating that strong immunity was induced and that immunological memory had been established. These protected mice showed vitiligo-like depigmentation on their skin, evidence of immunity to melanocytes, while mice vaccinated with the control plasmid did not (data not shown). These results demonstrate that fusion of GFP to CTL epitope broke the tolerance to tumour antigen. Tumour growth was also suppressed in mice vaccinated with a single intramuscular injection of pGFP-TRP-2 (data not shown).

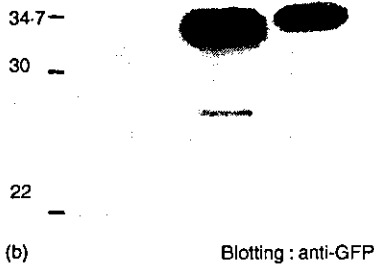
Cellular requirement for induction of antitumour immunity by vaccination with pGFP-TRP-2

It is known that tumour-specific CTLs play a crucial role in rejection of a tumour. We therefore carried out an



(a)

MW'000 COS-7 GFP GFP-TRP-2 TRP-2
51.8—



(b)

Figure 1. Plasmid construction and expression of GFP-TRP-2 in COS-7 transfectants. (a) Schematic representation of pGFP-TRP-2 (upper panel) and pTRP-2 (lower panel). (b) COS-7 cells were transfected with pGFP or pGFP-TRP-2 using Lipofectamine. Expression of GFP or GFP-TRP-2 was detected by Western blotting with anti-GFP as the first antibody. Sizes of the molecular weight markers are shown on the left. The experiment of (b) was repeated three times with similar results.

experiment to determine whether vaccination could induce CTLs specific for the tumour before the challenge. No activity of CTLs was detected in splenocytes of mice vaccinated with pGFP or even with pTRP-2. In contrast, splenocytes obtained from mice vaccinated with pGFP-TRP-2 showed prominent cytotoxicity to H-2^b-bearing EL4 pulsed with TRP-2₁₈₁₋₁₈₈ peptide (Fig. 3). Thus, vaccination with pGFP-TRP-2 effectively induced specific CTLs, indicating that activation of CD4⁺ T cells is crucial for induction of CTLs by this vaccination. No evidence of

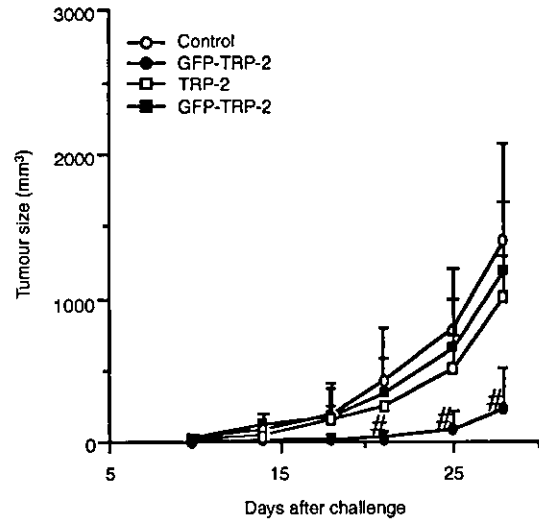


Figure 2. Induction of antitumour immunity against B16F1 in C57BL/6 mice by vaccination with pGFP-TRP-2. C57BL/6 mice were immunized with pGFP (open circles), pTRP-2 (open squares), pGFP-TRP-2 (closed circles) or pGFP plus pTRP-2 by using a gene gun and then challenged s.c. with 2×10^5 of B16F1 melanoma cells. Each value represents the mean \pm SD from seven mice. Tumour growth was measured twice a week after challenge. #*P* < 0.01 compared with the other two groups, by unpaired Student's *t*-test. This experiment was repeated five times.

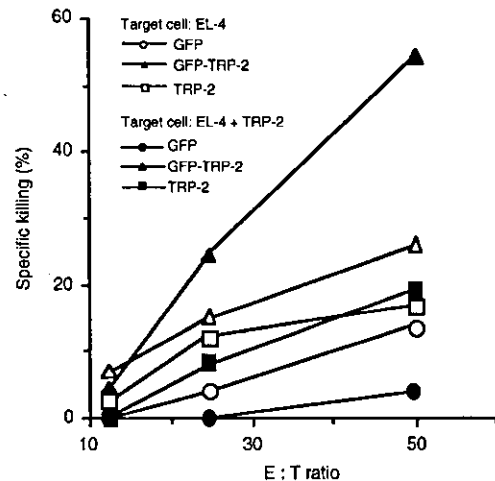


Figure 3. CTL activity induced by vaccination with pGFP-TRP-2. Effector cells were prepared from splenocytes (5×10^6) obtained from immunized C57BL/6 mice after *in vitro* stimulation with mitomycin C-treated B16F1 cells for 5 days. [³H]thymidine-labelled EL-4 cells were pulsed with (closed symbols) or without (open symbols) TRP-2₁₈₁₋₁₈₈ peptide used as target cells. Effector and target cells were incubated for 4-5 hr and then harvested and counted using a beta counter, and specific killing was calculated. This experiment was repeated for three times with similar results.

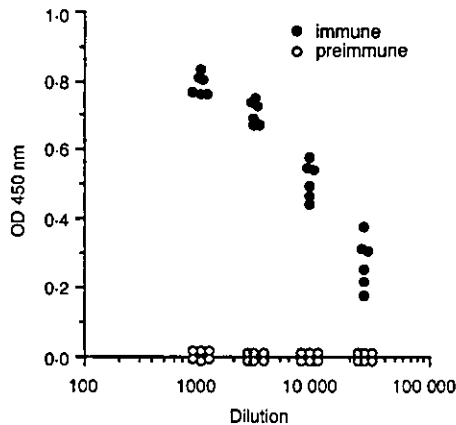


Figure 4. GFP-specific IgG in mice immunized with pGFP-TRP-2. C57BL/6 mice were immunized five times at 1-week intervals by using a gene gun. Serum samples collected from the immunized mice one week after the fifth immunization (closed symbols) were serially diluted, and ELISA was performed on plates coated with recombinant GFP. Sera from preimmune mice (open symbols) were also analyzed. Each symbol represents the result from an individual mouse. This experiment was repeated for three times.

CD4⁺ T cell responses to TRP-2 was obtained since the plasmid used for vaccination only expressed CTL epitope of TRP-2 (data not shown). However, a foreign protein, GFP, could activate CD4⁺ T cells as assessed by production of GFP-specific IgG. High levels of GFP-specific IgG antibodies were detected in sera obtained from mice vaccinated with pGFP-TRP-2 (Fig. 4). Because it is known that isotype switching requires help from antigen-specific CD4⁺ T cells, this finding indirectly confirmed the induction of GFP-specific CD4⁺ T cells *in vivo*, although we have no direct evidence that GFP recalls responses of CD4⁺ T cells *in vitro*.

We further investigated the cells responsible for antitumour immunity by carrying out cell-depletion experiments. Mice vaccinated with pGFP-TRP-2 were depleted of a specific cell subset by administration of the corresponding antibodies. After confirmation of successful depletion of the T-cell subset, B16F1 cells were inoculated. As shown in Fig. 5, depletion of CD8⁺ T cells abrogated the antitumour effect induced by vaccination with pGFP-TRP-2. Tumours in mice depleted of CD8⁺ T cells developed more rapidly than even those in unvaccinated mice. Depletion of CD4⁺ T cells also significantly affected the antitumour immunity but to a lesser extent than that of depletion of CD8⁺ T cells. Thus, both CD8⁺ and CD4⁺ T cells were required for induction of antitumour immunity.

Degradation of GFP-TRP-2 by proteasome

If a foreign GFP only functions as an activator of CD4⁺ T cells, coimmunization with two plasmids expressing either TRP-2 or GFP should be effective. However, our results demonstrated that coimmunization did not induce CTL

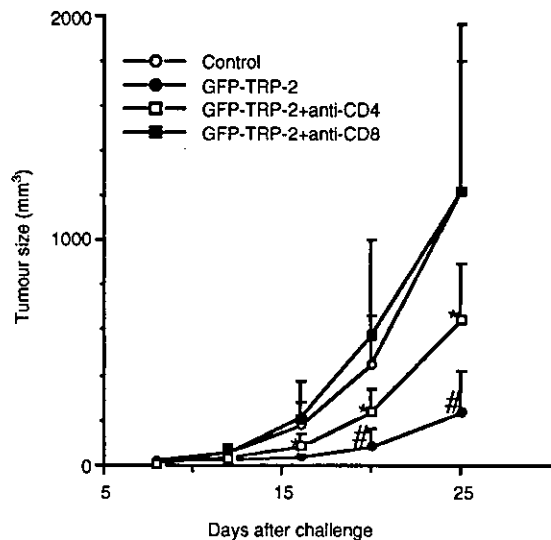


Figure 5. Cellular requirement for antitumour immunity induced by immunization with pGFP-TRP-2. Twenty-one C57BL/6 mice immunized with pGFP-TRP-2 were divided into three groups, one of which was left untreated (closed circles). Two groups of mice were administered with either anti-CD4 (open squares) or anti-CD8 mAb (closed circles) on days -3, -1, 1 and 3. Tumour cells were challenged on day 0. Unvaccinated mice (open circles) were also challenged. Each value represents the mean \pm SD from seven mice. # $P < 0.01$ compared with control and anti-CD8 group and * $P < 0.05$ compared with untreated immune group, by unpaired Student's *t*-test. This experiment was repeated three times with similar results.

activity (Fig. 2), strongly suggesting the requirement of a physical linkage between the two proteins and an additional role of GFP in induction of CTLs. We therefore hypothesized that intracellular GFP is rapidly degraded by the ubiquitin-proteasome pathway and the TRP-2₁₈₁₋₁₈₈ fused to GFP is readily cut off from GFP and efficiently presented to MHC class I molecules, resulting in effective induction of CD8⁺ T specific for the CTL epitope. Therefore, the CTL epitope should effectively associate with MHC class I molecule and then be presented to CD8⁺ T cell. To prove this, we transfected COS-7 cells with pGFP or pGFP-TRP-2 in the presence of a proteasome inhibitor MG-132 (Fig. 6). Chemical inhibition of proteasome resulted in accumulation of both GFP alone and an epitope fused to GFP, indicating that intracellularly expressed intracellular GFP is susceptible to proteasomal degradation. Thus, antigen presentation of TRP-2₁₈₁₋₁₈₈ to CD8⁺ T cells became significantly effective by fusion to the foreign protein GFP.

Defective induction of antitumour immunity in PA28 α/β ^{-/-} mice by immunization with pGFP-TRP-2

In order to clarify the role of the proteasome in antitumour immunity induced by pGFP-TRP-2, we employed PA28 α/β ^{-/-} mice, because TRP-2₁₈₁₋₁₈₈ is cut off by PA28-

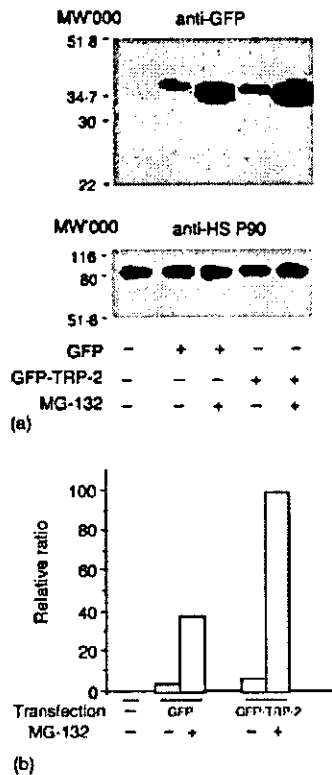


Figure 6. Processing of GFP and GFP-TRP-2 protein by proteasome. COS-7 cells were transfected with pGFP or pGFP-TRP-2 using Lipofectamine. Twenty-four hr after transfection, the proteasome inhibitor MG-132 was added and cells were prepared 24 hr later for detection of GFP and HSP90 by using Western blot. Sizes of molecular weight markers are shown on the left in kilodaltons (a). Relative expression of GFP-TRP-2 to that of HSP90 was calculated densitometrically (b). This experiment was repeated twice.

mediated ubiquitin-proteasome pathway.¹⁵ PA28 $\alpha/\beta^{-/-}$ mice immunized with pGFP-TRP-2 were markedly susceptible to B16F1 tumour challenge compared with wild-type B6 mice immunized with the same plasmid (Fig. 7a). On day 14 after s.c. challenge with B16F1, these mice were challenged again with intravenous (i.v.) injection of B16F10 tumour cells, and the number of lung metastases of B16F10 cells was counted three weeks after the i.v. challenge. The number of lung metastases was smaller in pGFP-TRP-2-immunized mice than in control mice ($P = 0.02$). The number of lung metastases in PA28 $\alpha/\beta^{-/-}$ mice immunized with the same plasmid was slightly less than that in control mice ($P = 0.15$) but remarkably greater than that in pGFP-TRP-2-immunized mice ($P = 0.06$), although the difference was not significant (Fig. 7b). Coincident with the results of tumour growth, CTL activity against the TRP-2₁₈₁₋₁₈₈ epitope was lower in PA28 $\alpha/\beta^{-/-}$ mice immunized with pGFP-TRP-2 than in wild-type mice (Fig. 7c). Similar levels of GFP-specific IgG were detected in PA28 $\alpha/\beta^{-/-}$ and wild-type B6 mice after three immunizations with pGFP-TRP-2 (Fig. 7d), indicating that the

responses of CD4⁺ T cells were not affected by PA28 α/β deficiency. These results proved that the proteasome activated by PA28 plays an important role in the induction of TRP-2₁₈₁₋₁₈₈-specific CTL and antitumour immunity following DNA vaccination with pGFP-TRP-2.

DISCUSSION

The final goal for tumour immunotherapy is to induce antigen-specific CTL activity. A major obstacle that must be overcome for induction of effective tumour immunity is peripheral self-tolerance to tumour antigens. For this purpose, we constructed a plasmid (pGFP-TRP-2) encoding a fusion protein of a defined CTL epitope, TRP-2₁₈₁₋₁₈₈, to a foreign protein, GFP. GFP is known to be readily targeted by ubiquitin-fusion degradation (UFD) pathway, a virtual route of the ubiquitin-proteasome pathway.¹⁴ Furthermore, GFP has a potential to activate specific CD4⁺ T cells.¹⁸ In the present study, immunization with pGFP-TRP-2 induced potent antitumour immunity promoted by both CD4⁺ and CD8⁺ T cells. Our vaccination with the naked chimeric DNA clearly demonstrated the crucial contributions of the UFD pathway to the antitumour immunity, as evaluated by experiments *in vitro* with proteasome inhibitor MG-132 and *in vivo* with proteasome activator PA28 α/β knockout mice.

It is noteworthy that GFP fused to TRP-2₁₈₁₋₁₈₈ expressed within cytosol of APC plays a central role in the induction of TRP-2₁₈₁₋₁₈₈-specific CTLs. Thus, directed linkage between a CTL epitope and GFP should be required to induce TRP-2₁₈₁₋₁₈₈-specific CTLs, since DNA vaccination with pGFP plus pTRP-2 was not effective in inducing the antitumour immunity, as shown in Fig. 2. One interpretation for the necessity of direct linkage between GFP and TRP-2 is that either MHC class II-binding peptide, GFP recognized by CD4⁺ T cells, or MHC class I-binding peptide, TRP-2₁₈₁₋₁₈₈ recognized by CD8⁺ T cells, have to be presented by the same APC. Notably, cytosolic foreign proteins which have been monoubiquitinated are processed by lysosomal enzymes.¹⁹ Accordingly, some peptides in those foreign proteins should be presented by MHC class II molecules, which activate specific CD4⁺ T cells. Another possibility should be that a part of the fusion protein, GFP-TRP-2, generated within APC/DC is secreted into circulation and then pinocytosed/phagocytosed by other APC/DC and then some degraded peptides of GFP should be presented by MHC class II molecules on the APC/DC resulting in an inevitable activation of GFP-specific CD4⁺ T cells, while TRP-2₁₈₁₋₁₈₈ fused to GFP stimulates its specific CD8⁺ T cells. As shown in Fig. 1, GFP is susceptible to degradation by proteasome, the initial step of antigen presentation to CD8⁺ T cells. Endogenous antigens such as tumour antigens is processed by proteasome after ubiquitination.² Ubiquitin binds target proteins via lysine.³ GFP contains 19 lysine residues,²⁰ and is easily degraded by the UFD pathway,¹⁴ which may be very helpful for ubiquitination and antigen processing of the fusion protein GFP-TRP-2₁₈₁₋₁₈₈. Thus, the ubiquitin-proteasome

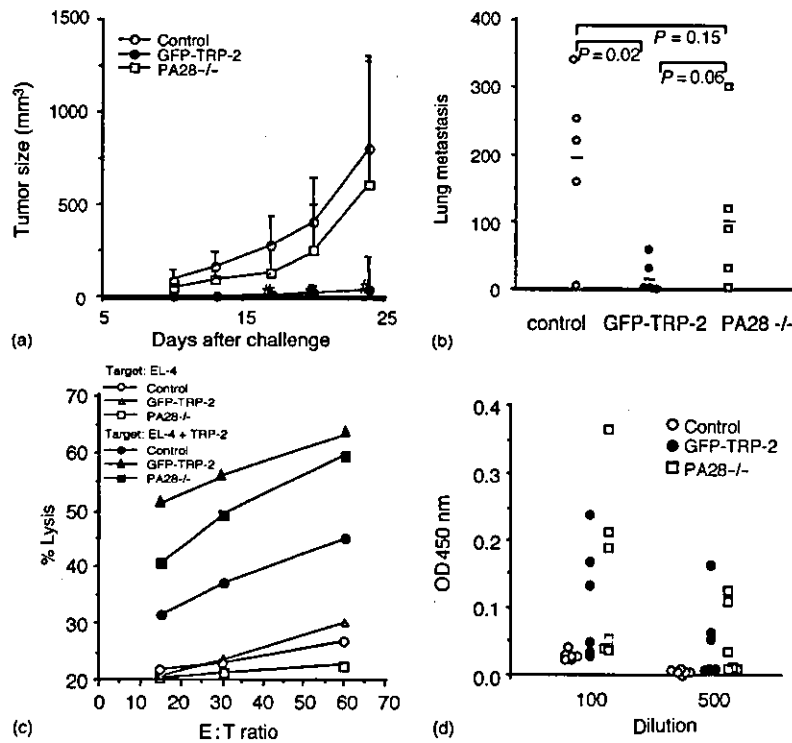


Figure 7. Defective induction of antitumour immunity by immunization with pGFP-TRP-2 in PA28 $\alpha/\beta^{-/-}$ mice. C57BL/6 (closed circles) and PA28 $\alpha/\beta^{-/-}$ knockout mice (open squares) immunized with pGFP-TRP-2 as well as unvaccinated control mice (open circle) were challenged s.c. with 2×10^5 B16F1 cells, and tumour growth was investigated (a). Number of lung metastases in mice immunized with pGFP-TRP-2 (b). CTL activity in pGFP-TRP-2-immunized B6 and PA28 $\alpha/\beta^{-/-}$ mice (c). GFP-specific IgG was detected by ELISA in pGFP-TRP-2-immunized B6 and PA28 $\alpha/\beta^{-/-}$ mice (d). Each value represents the mean \pm SD from seven mice. # $P < 0.01$ compared with the other two groups, by unpaired Student's t -test. This experiment was repeated three times with similar results.

pathway¹ should play an indispensable role in the present vaccination system. These results agree with the report by Steitz *et al.*¹⁸ They showed that immunization with a plasmid encoding a fusion protein of GFP and mTRP-2₃₀₋₅₁₉ could induce tumour-specific protective immunity promoted by TRP-2₁₈₀₋₁₈₈-specific CD8⁺ T cells as evaluated by interferon- γ production through being activated by GFP-specific CD4⁺ T cells, although they did not assay CTL activity. In their experiments using adenovirus vector encoding the fusion protein, effective antitumour immunity against lung metastasis of B16 melanoma was induced.

Many melanoma tumour antigens have been identified in the last decade because of the development of new technology. A solid base for antigen-specific cancer immunotherapy has been established by approaches such as immunization with antigenic peptides,²¹ immunization with dendritic cells pulsed with tumour antigens,²² and immunization with a naked DNA or recombinant viral vaccines.²³ Immunization with plasmid DNA has some advantages compared with other approaches: it is safe, readily deliverable, molecularly defined and can easily be constructed to enhance antigenicity by various means such as enhancement of antigen processing or adjuvant activity through a

CpG motif included in plasmid DNA. We demonstrated here that vaccination of mice with a naked chimera DNA expressing a melanoma CTL epitope fused to GFP conferred protection against the tumour challenge and effectively induced epitope-specific CTLs. Immunization with DNA encoding peptide alone often failed to induce antitumour immunity effectively. The cause of this failure is still under investigation, although there exists at least four possible mechanisms. First, linked T-cell help may be required. Second, an N-terminal extension of a peptide epitope may simply prolong its biological half life. Third, cross-presentation of the construct by dendritic cells may require an antigen larger than the peptide epitope itself for either targeting to the processing pathway. Fourth, the N-terminus of the CD8⁺ T-cell epitope may protect from degradation.

Taken together, our results indicate that fusion of a foreign protein to a CTL epitope plays dual roles in the induction of effective antitumour immunity. One role is activation of CD4⁺ T cells that are required for induction of fully functional CD8⁺ T cells, and the other role is facilitation of antigen presentation to CD8⁺ T cells through the ubiquitin-proteasome pathway. This vaccine strategy using CTL epitopes fused with foreign proteins

would be applicable to human immunotherapy. It is required, however, to seek suitable foreign proteins that are readily processed by the UFD pathway.

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Photosensitive Drug Eruption Induced by Efavirenz in a Patient with HIV Infection

Key words: photosensitive drug eruption, efavirenz, HIV infection

HIV infection causes various acute and chronic cutaneous disorders such as rash, seborrheic dermatitis-like eruption, alopecia, herpes simplex and Kaposi's sarcoma, some of which are associated with immunosuppression. HIV infection down-regulates various types of functional properties of CD4+ helper and regulatory T cells, leading to the devastating immunosuppression in the majority of infected hosts. But HIV infection sometimes induces immunoenhancing features during its clinical course, probably because of the deterioration of the regulatory T cell function. Individuals with AIDS/HIV infection are indeed at higher risk of developing adverse drug reactions (1). Multiple drugs are usually prescribed to the patients with AIDS/HIV infection for preventing the replication of HIV and for the treatment of the associated infectious diseases. In patients with AIDS/HIV infection, the adverse drug reaction often becomes very severe, developing life-threatening toxic epidermal necrolysis and Stevens-Johnson's syndrome (2). It is now known that high CD8+ cell count and age less than 36 years indicate a risk of drug eruption [respective odds ratios: 3.5 (95% CI 1.6–7.8), $p=0.002$, and 2.1 (95% CI 1–4.6), $p=0.06$] (3).

In this issue, Yoshimoto et al reported the first Japanese patient with HIV infection who developed a photosensitive drug eruption induced by efavirenz, a non-nucleotide reverse transcriptase inhibitor (4).

See also p 630.

The patient presented a pruritic edematous erythema on sun-exposed areas such as face, neck and bilateral forearms after playing golf. The photo patch test revealed a positive reaction to efavirenz under exposure to ultraviolet A light. Lopinavir/ritonavir was substituted for efavirenz, and the patient never experienced photosensitivity after the discontinuation of efavirenz. The photosensitivity very much decreases the quality of life. Physicians should be aware of the possibility of photosensitive drug eruption during antiviral treatment for AIDS/HIV infection.

Masutaka FURUE, MD

Department of Dermatology, Graduate School of Medical Sciences,
Kyushu University, 812-8582

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Letter to the Editor

Bilateral epiretinal membranes in nevoid basal cell carcinoma syndrome

Shigeo Yoshida,¹ Hiroshi Yoshikawa,¹ Ayako Yoshida,¹ Takehiko Nakamura,¹ Yoshihiro Noda,¹ Hisaki Gondoh,² Shuji Fukagawa,² Yoichi Moroi,² Kazunori Urabe,² Masutaka Furue² and Tatsuro Ishibashi¹

¹Department of Ophthalmology, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan

²Department of Dermatology, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan

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Sir,

Nevoid basal cell carcinoma syndrome (NBCCS) is a dominant, autosomal disorder linked to chromo-

some 9q22.3-q31 and is caused by mutations of the patched homologue (PTCH) gene (Gorlin & Goltz 1960;

Hahn et al. 1996). PTCH mutations can be tumorigenic and cause developmental anomalies by affecting the sonic

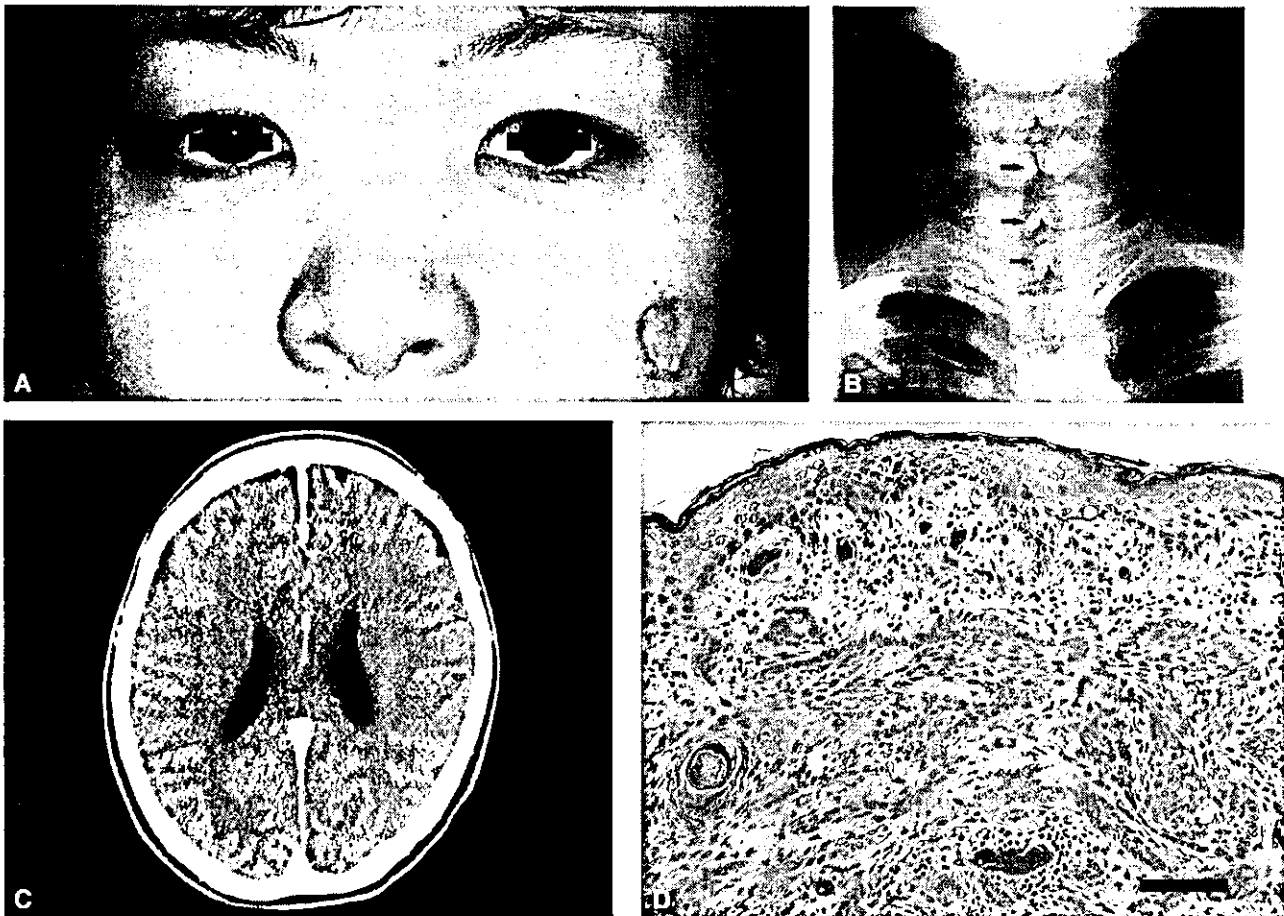


Fig. 1. (A) Photograph of the patient at age 27 years showing an ulcerating mass measuring 2.0 × 2.0 cm on the left cheek. A marked hypertelorism can also be noted. (B) Chest radiograph showed bifid cervical vertebrae, some of which are indicated by arrows. (C) Computed tomographic scan of the patient's head showing calcification of the falx cerebri. (D) Histological section of the specimen removed from the cheek showing proliferation of basaloid cells with severe atypia in the fibrous dermis. Parts of the nests show peripheral nuclear palisading. Focal keratinization is also seen. These findings are characteristic of BCC.

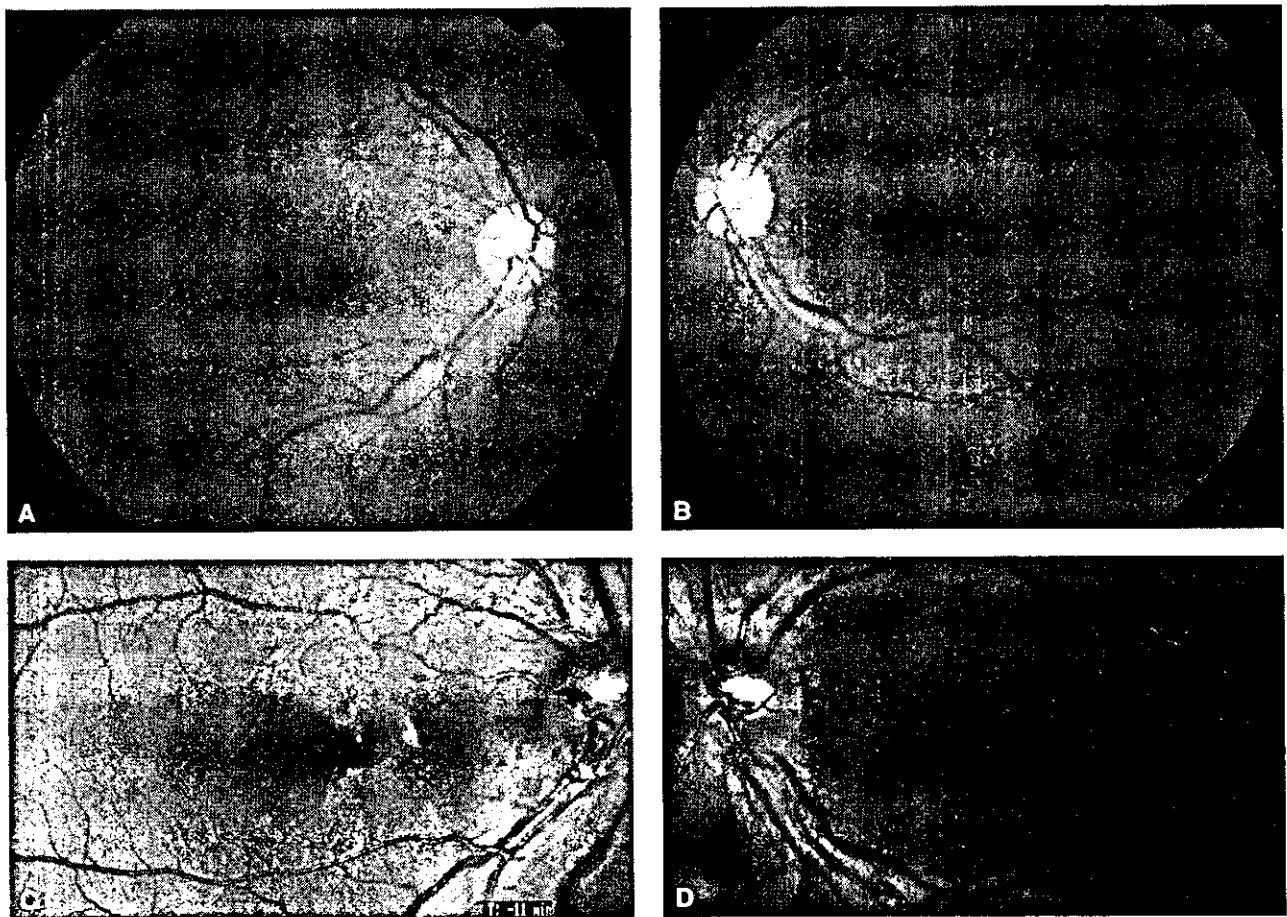


Fig. 2. (A, B) Fundus photographs. Both right and left eyes show a white preretinal membrane. In the left fundus, a distortion of the retinal vasculature can be seen. (C, D) Confocal images of the right and left fundus obtained by scanning laser ophthalmoscope. A well demarcated dense epiretinal membrane is observed above the fovea with radial folds of the internal limiting membrane.

hedgehog/patched signalling pathway (Hahn et al. 1999). This syndrome is characterized by multiple basal cell carcinomas (BCCs), odontogenic keratocysts of the jaw, congenital skeletal anomalies, ectopic calcification, palm and/or sole pits, and ocular abnormalities (Kimonis et al. 1997). The ocular abnormalities include hypertelorism, strabismus, eyelid papilloma, medullated nerve fibres, and hamartoma of the retina (De Potter et al. 2000).

The presentation features of this syndrome are variable both within and between families (Black et al. 2003). We describe a case of NBCCS with bilateral epiretinal membranes (ERMs).

A 27-year-old Japanese woman was seen at Kyushu University Hospital. She had had a tumour on her left cheek (Fig. 1A) and another on her right lower abdomen since the age of 25 years. She also had hypertelorism (Fig. 1A), bifid vertebrae (Fig. 1B), jaw

cysts, and palmar/plantar pits. A computed tomographic scan showed intracranial calcification of the falx cerebri (Fig. 1C). She was operated on for the jaw cysts, and the lesions on her cheek and abdomen were removed. A diagnosis of BCC was made from the histopathological findings of the specimens (Fig. 1D).

Her family history revealed that both her father and brother had odontogenic keratocysts of the jaw and that her grandfather had undergone a tumour resection, the histological findings of which are unknown. Based on the family history and the systemic features, a diagnosis of NBCCS was made.

Ophthalmologically, the subject's best corrected visual acuity was 20/25 OD and 20/20 OS, and the anterior segments of both eyes were unremarkable. Fundus examination showed an ERM around the fovea in both eyes

(Fig. 2A, B). Scanning laser ophthalmoscopy with an argon blue laser (Rodensstock Instruments, Munich, Germany) revealed a well defined, fibrous-like tissue with folds of the internal limiting membrane (Fig. 2C, D). There was no evidence of a posterior vitreous detachment.

Epiretinal membranes are a common finding in elderly patients but are rare in young patients. Juvenile ERMs are usually unilateral and occur as secondary phenomena, for example after ocular trauma, ocular toxocariasis, Coats disease and pars planitis (Benhamou et al. 2002). In our case, none of these findings was present. We also carefully searched for a hamartoma of the retina and retinal pigment epithelium (RPE) by fluorescein and indocyanine green angiography. No hamartoma was found. Thus, we suggest that this was a case of primary ERM.

It has been recently reported that the retinas of *PtchlacZ+/-* mice exhibit Muller cell-derived gliosis and that, in NBCCS patients, the intraretinal glial response results in ERM formation (Black et al. 2003). This report and our own case lead us to believe that, rather than being coincidental, the ERMs instead represent an intraocular manifestation of NBCCS, and may have developed because of the tumorigenic condition induced by the abnormal hedgehog/patched signalling caused by the *PTCH* mutation. Molecular analysis of the *PTCH* gene and phenotype-genotype correlations might thus provide insights into the mechanisms responsible for the ERM in cases of NBCCS. We recommend a careful fundus examination in patients with NBCCS even if they have good visual acuity.

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Correspondence:

Shigeo Yoshida MD, PhD
 Department of Ophthalmology
 Kyushu University
 Graduate School of Medical Sciences
 Fukuoka 812-8582
 Japan
 Tel: + 81 92 642 5648
 Fax: + 81 92 642 5663
 Email: yosida@med.kyushu-u.ac.jp