Exp Dermatol 1995: 4: 20–29
Printed in Denmark · All rights reserved

Experimental Dermatology
ISSN 0906-6705

Low ICAM-1 expression in the epidermis of depigmenting C57BL/6J-mi^{vit}/mi^{vit} mice: A possible cause of muted contact sensitization

Nordlund JJ, Csato M, Babcock G, Takei F. Low ICAM-1 expression in the epidermis of depigmenting C57BL/6J-mi^{vit}/mi^{vit} mice: A possible cause of muted contact sensitization.
Exp Dermatol 1995: 4: 20–29. © Munksgaard, 1995

Abstract: The depigmenting C57BL/6J-mi^{vit}/mi^{vit}) (mi^{vit}/mi^{vit}) mouse, a congenic mutant of the C57BL/6 strain, exhibits an isolated, single immune deficiency. It is unable to mount a normal immune/inflammatory response upon epicutaneous application of DNFB or TNCB, although it does respond normally to oxazalone. The present investigtions have been carried out to further study this deficiency. *In vivo*, C57BL/6 mice could be sensitized by the epicutaneous application of the hapten TNCB, the subcutaneous injection of hapten(TNBS)-conjugated C57BL/6, and hapten conjugated mi^{vit}/mi^{vit} epidermal cells. In the mi^{vit}/mi^{vit} mice, however, only subcutaneous injection of haptenized C57BL/6 epidermal cells caused an immune response. The response of these mi^{vit}/mi^{vit} mice could be documented only by adoptive transfer of splenic lymphocytes into naive C57BL/6 animals which then reacted to challenge doses of TNCB. These observations suggest that mi^{vit}/mi^{vit} epidermal cells can process and present and mi^{vit}/mi^{vit} T lymphocytes can react to the antigen. We postulated the presence of a deficient *in vivo* interaction between epidermal cells and T lymphocytes in the mi^{vit}/mi^{vit} mice. ICAM-1 is an important adhesion signal regulating epidermal cell/T-lymphocyte interaction. Its expression in mi^{vit}/mi^{vit} mice was studied using YN1/1 antibody against MALA-2, the murine counterpart of human ICAM-1. In contrast to C57BL/6 animals, the mi^{vit}/mi^{vit} epidermis essentially did not stain with the antibody after hapten challenge. *In vitro* after stimulation with TPA or IFN-γ, the mi^{vit}/mi^{vit} epidermal cells expressed significantly lesser amounts of ICAM-1 than the C57BL/6 epidermal cells. Lower expression of ICAM-1 by mi^{vit}/mi^{vit} epidermal cells has also been demonstrated both by direct staining and by flow cytometry. The binding of lymphocytes to mi^{vit}/mi^{vit} epidermal monolayers, which were stimulated to express ICAM-1 by IFN-γ, was decreased compared to that of C57BL/6 epidermal cells. We conclude that the muted contact sensitization response detected *in vivo* in the mi^{vit}/mi^{vit} mice at least partly results from lower expression of ICAM-1 and thus defective epidermal cell/T-lymphocyte interaction.

James J. Nordlund, Miklos Csato, George Babcock† and Fumio Takei*

University of Cincinnati College of Medicine, Department of Dermatology, and †Department of Surgery, Shriners Burns Institute, Cincinnati, Ohio; and *Terry Fox Laboratory, BC Cancer Research Centre, Vancouver, British Columbia, Canada

Key words: vitiligo – adhesion molecules – contact dermatitis

James J. Nordlund, M.D., University of Cincinnati College of Medicine, Department of Dermatology, 231 Bethesda Avenue, Cincinnati, OH, U.S.A. 45267-0592

Accepted for publication 11 July 1994

Introduction

We have reported previously that the C57BL/6J-mi^{vit}/mi^{vit} (mi^{vit}/mi^{vit}) mouse, a congenic mutant of the C57BL/6 strain, exhibits an isolated, single immune deficiency (1, 2). The mutant animals are unable to mount a normal immune/inflammatory response upon epicutaneous application of potent

Supported in part by NIG Grant AM25252, NIOSH Grant OH02091, The Ahlers Foundation, Cincinnati, Ohio, The National Vitiligo Foundation, Tyler Texas, and the University of Cincinnati General Clinical Research Center Grant #M01-RR00068 (NIH).
This work was presented in part at the ESDR-ISID-SID Tricontinental Meeting, Washington, DC, May 2–5, 1990; and appeared in abstract form (J Invest Dermatol 1990: 94: 516).

contact allergens such as dinitrofluorobenzene (DNFB), or trinitrochlorobenzene (TNCB) (1, 2), although they do respond to oxazalone. Other cellular immune parameters such as the dermal-type delayed hypersensitivity response, cutaneous allograft and tumor rejection, and antibody generation to both particulate and soluble antigens in the mi^{vit}/mi^{vit} mice were similar to those responses of C57BL/6 controls (2, 3). These results suggest that the defect is unique to certain antigens administered by certain routes. The deficient contact hypersensitivity responsiveness could be restored with skin obtained from the C57BL/6 mice and transplanted to the mi^{vit}/mi^{vit} mice. Sensitization did occur when the DNFB was applied to the C57BL/6 graft. This suggest that, once sensitized, epidermal murine cells function normally to challenge by the allergen. In contrast, mi^{vit}/mi^{vit} skin on the C57BL/6 mouse did not sensitize (2). From these observations, it had been postulated earlier that the deficiency resides in part in the afferent limb of the immune response and within the epidermis of the mi^{vit}/mi^{vit} animals.

The epidermis of the mi^{vit}/mi^{vit} animals is different from that of the C57BL/6 mouse (1, 2, 4). The animals are born with melanocytes which are near normal in number, but there is gradual loss of epidermal melanocytes in the mi^{vit}/mi^{vit} animals. By 6 to 12 months of age there are few melanocytes in the epidermis (4). Light and EM studies confirm that the loss of melanocytes is not associated with an inflammatory response in the skin (1). There is a slight decrease in Ia-positive Langerhans cells and an increase in Thy-1 positive dendritic epidermal cells observed by 6 months of age. The loss of contact sensitization response seems to be associated with loss of melanocytes. The immune deficiency in the mi^{vit}/mi^{vit} mice resembles a similar defect in humans with vitiligo who also exhibit a decreased contact sensitization response on depigmented skin (5, 6).

We have studied the muted responsiveness to contact allergens on the assumption that it was caused by dysfunction of epidermal cells and/or T lymphocytes. Data outlined below indicate that both the antigen presentation by the epidermis and hapten recognition by the T cells is sufficient in the mi^{vit}/mi^{vit} mice. The mi^{vit}/mi^{vit} epidermis is deficient, however, in the expression of intercellular adhesion molecule-1 (ICAM-1), demonstrated both *in vivo* and *in vitro*. The deficient/aberrant expression of ICAM-1 might result in insufficient trafficking of T lymphocytes or Lagerhans cells or out of the epidermis, or in an epidermal environment ineffective for sensitization. We propose that deficient epidermal cell/T-cell interaction caused by lower expression of ICAM-1 is responsible in part for the muted contact sensitivity in the depigmented animals.

Material and methods

Animals

The C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). C57BL/6J-mi^{vit}/mi^{vit} mice were reared in the AALAC approved animal facility at the University of Cincinnati College of Medicine. Mi^{vit}/mi^{vit} mice at the age of 6 weeks have a black pelage except for a piebald band on the ventral thorax and the nape of the neck, and have only a minimal loss of epidermal and follicular melanocytes. At 24 wk of age they are almost totally white and have only a very few epidermal or ocular melanocytes (4). Mi^{vit}/mi^{vit} mice at the age of 6–8 months were used throughout the experiments. At least 4 animals, and usually 6, were used in each group in each experiment. For all experiments, the normal and mutant mice were of the same age.

Preparation of cell suspensions

Epidermal cell suspensions were prepared by trypsin digestion. Briefly, ears from anesthetized mice were excised, spit mechanically into ventral and dorsal halves and incubated overnight at 4°C with 0.25% trypsin (Gibco, Grand Island, NY). The epidermis was then removed and incubated with 0.025% DNAse (Sigma Chemical Co., St. Louis, MO) for 5 min. The cells were filtered and washed three times in 2% FCS-HBSS (Gibco, Grand Island, NY). The single-cell suspensions were used for studies on hapten conjugation and used in sensitization studies, or for *in vitro* studies on expression of ICAM-1 by cells stimulated by IFN-γ.

Adoptive transfer of splenocytes

Spleen cells from naive mice or mice exposed to allergen by various methods were prepared by teasing spleens with a glass rod in HBSS followed by filtering through a nylon mesh (Tetko, Inc., Elmsford, NY). The cells were then washed three times in HBSS and the red blood cells were lysed in NH_4Cl-Tris buffer. The unfractionated lymphocytes were then layered onto Lympholite, [density 1.087 (Accurate Chemical Company, Westbury, NY)], and centrifuged for 30 min at 25°C. The interface containing the lymphocytes was then washed twice and used in the adoptive transfer experiments.

Preparation of TNBS-conjugated epidermal cells

For some sensitization studies, epidermal cells were hapten-conjugated. Briefly, after washing, the epidermal cells in single-cell suspension were incu-

bated with 1 mM 2,4,6-trinitrobenzene sulfonic acid (TNBS) (Aldrich Chemical Co., Milwaukee, WI) in HBSS for 10 min at 37°C. The cells were then washed three times in 10% FCS-HBSS and used in the above studies.

Sensitization for contact sensitivity and challenge

Mice of same age were sensitized by epicutaneous application of 2,4,6-trinitrochlorobenzene (TNCB) [(Easteern Chemical, Smithdown, NY) 100 µl of 7% TNBC in acetone:olive oil] to the razor-shaved abdomen. In other experiments, mice were sensitized by subcutaneous injection of 10^6/100 µl TNCB-conjugated viable syngeneic epidermal cells into the dorsal skin. Six days after painting of the skin or after the subcutaneous injection of the mice, the mice were challenged on the ears with a total of 20 µl of 1% TNCB in acetone:olive oil (4:1). Ear thickness was measured with an engineer's micrometer immediately before and 24 hours after the challenge. The increment of ear thickness was calculated as a measure of the contact sensitization response. Mice receiving challenge only were included as irritant controls. These experiments have been repeated on numerous occasions with very reproducible results as published previously (1, 2).

In adoptive transfer of contact sensitivity, splenic lymphocytes were prepared either from mice exposed to allergen by epicutaneous application; or by subcutaneous injection with haptenized epidermal cells. The splenic lymphocytes were transferred intravenously (i.v.) into naive mice (2×10^7 viable lymphocytes/100 µl/mouse), and immediately after the transfer the recipient mice were challenged with 1% TNCB on the ears. The ear swelling was measured before challenge and at 24 h. Experiments were repeated at least twice with very similar results. All mice were of the same age.

Immunohistochemistry

Full thickness ear biopsies were obtained from C57BL/6 and mi^{vit}/mi^{vit} mice under anesthesia 48 h after challenge with hapten. Six-micron cryostat sections were fixed in acetone at 4°C for 10 min. The sections were incubated in 3% hydrogen peroxide-methanol to block endogenous peroxidase and coated with PBS containing 1.5% normal rabbit serum for 20 min. The sections were treated with undiluted hybridoma culture supernatant (rat anti-murine YN1/1 antibody recognizing MALA-2, the murine counterpart of human ICAM-1) (7, 8, 9) for 90 min at 37°C. After washing, the sections were incubated for 30 min with biotinylated rabbit anti-rat IgG diluted in PBS followed by a 45-min incubation with avidin-bionylated peroxidase complex (Vectastain Standard Kit, Vector Laboratories, Burlingame, CA). The tissue was then reacted for 10 min with 3,3′-diaminobenzidine tetrahydrochloride solution with hydrogen peroxide. The sections were counterstained with 1% toluidine blue-azure-borax and mounted.

Immunocytochemistry

After trypsinization, suspensions of epidermal cells from C57BL/6 or from mi^{vit}/mi^{vit} mice were maintained in MCDB 153 medium (Irving Scintific, Santa Ana, CA) supplemented with 5 ng/ml EGF (Collaborative Research, Waltham, MA), 5 µg/ml insulin (Sigma Chemical Co., St. Louis, MO), 1.4 µM hydrocortisone (Sigma), 0.2% bovine pituitary extract (Clonetics, San Diego, CA), 0.5% FCS and antibiotics and antimycotic for 72 h. ICAM-1 expression on epidermal cells was induced either by addition to the cultures for 24 h of 50 ng/ml 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (Sigma) or by 400 U/ml recombinant murine interferon-gamma (IFN-γ) (Genzyme, Boston, MA) (specific activity: 4.5×10^6 units/mg). Cells were then fixed with methanol for 10 min at 4°C. Immunoperoxidase staining for murine ICAM-1 was then performed as described for immunohistochemistry.

Flow cytometry

For flow cytometry, epidermal cells were maintained in culture for 72 h. One hundred U/ml of recombination murine IFN-γ (Genzyme, Boston, MA) was added to half of the cultures for the last 24 h. Epidermal cells were removed from the dishes with trypsin which was neutralized with 10% FCS. The cells were then washed with PBS with 1% bovine serum albumin (BSA) and 0.5% sodium azide at 4°C. Rat anti-murine YN1/1 antibody (100 µl) was added to the cells for 20 min at 4°C. After washing, the cells were labeled with FITC-labeled goat-anti-rat secondary antibody (Kirkegaard and Perry Lab, Inc.). After repeated washing the cells were fixed with 1% paraformaldehyde in PBS and subjected to flow cytometry on an Epics 753 (Coulter Electronics) using the 488 line of the argon ion laser for excitation. Emissions were collected using a 525 nm bands pass filter and histograms were analyzed using Easy 2 software (Coulter Electronics). Control (isotypic) cell populations were used to set electronic gates such that no more than 1% of the control cells were in the positive region.

Table 1. Binding of Mi^{vit}/mi^{vit} lymphoblasts to epidermal cells

Epidermis Cells	C57BL/6	(n)	Mi^{vit}/mi^{vit}	(n)
Untreated	17.25±9.40	(4)	14.75±8.01	(4)
IFN-γ	54.60±4.92*	(5)	20.80±5.90**	(5)
IFN-γ+YN1/1 antibody+	39.00±4.50	(3)	17.60±4.18	(3)
IFNγ+IgG 2b	18.60±5.60	(3)	16.30±6.75	(3)

Subconfluent monolayers of epidermal cells were treated with 400 U/mol recombinant murine IFN-γ for 24 hours. Concanavalin A-stimulated mi^{vit}/mi^{vit} lymphoblasts were incubated with the monolayers for 1 hour and lymphocyte binding was calculated. Results are mean±S.D. percent binding.

* Significantly different from similarly treated mi^{vit}/mi^{vit} ($p<0.0005$) and YN1/1 antibody-treated C57BL/6 epidermal cells ($p<0.005$).

** Mi^{vit}/mi^{vit} untreated vs. IFN-γ-treated not significant ($t=1.310$).

+ 250 µl undiluted hybridoma culture supernatant was added to the epidermal cells simultaneously with the lymphoblasts.

Epidermal cell-lymphocyte binding assay

Epidermal cells from C57BL/6 or mi^{vit}/mi^{vit} mice were grown as for immunocytochemistry and for flow cytometry in 35-mm tissue culture dishes (Falcon, Becton Dickinson) for 72 h. Two$\times 10^6$/2 ml epidermal cells were seeded per dish. No differences in the growth rate and pattern of growth between C57BL/6 and mi^{vit}/mi^{vit} cultures were noted. Half of the cultures from normal and mi^{vit}/mi^{vit} were treated with 400 U/ml recombinant murine IFN-γ (Genzyme, Boston, MA) for the last 24 h of culture. The culture medium was then aspirated and Con A-stimulated splenic lymphoblasts [2×10^7 spleen cells stimulated with 3 µl/ml Con A in RPMI 1640 with 10% FCS, 4 mM L-glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids and antibiotics (all from Gibco, Grand Island, NY) for 3 days] prepared from mi^{vit}/mi^{vit} mice were added to the dishes containing subconfluent (80% confluency) cultures of epidermal cells. In some studies on specificity, YN1/1 antibody (250 µl of undiluted hybridoma culture supernatant) was added to the epidermal cell cultures simultaneously with the Con A-lymphoblasts to block adherence of lymphocytes to ICAM-1. For the latter studies, control cultures were treated with isotype-matched IgG 2b rat antibody. The dishes were incubated for 1 h at 37°C in humidified air with 5% CO_2 and after gentle washing, the non-adherent lymphocytes were collected. The percentage of lymphocyte binding was calculated via the following formula:

$$\text{Percent binding} = \frac{\text{total No lymphocytes added} - \text{No non-adherent lymphocytes}}{\text{total No lymphocytes added}} \times 100$$

Binding studies were done on 3 to 5 control and experimental cultures (Table 1). Both control and experimental cultures were prepared and studies done simultaneously to avoid the variability that frequently is observed in sequential experiments.

Statistical analysis

Student's t-test was used for statistical analysis.

Results

Contact sensitization: Direct and adoptive transfer of splenic lymphocytes

In these experiments, as in prior studies in this and other laboratories (1, 2, 4), we noted the loss of

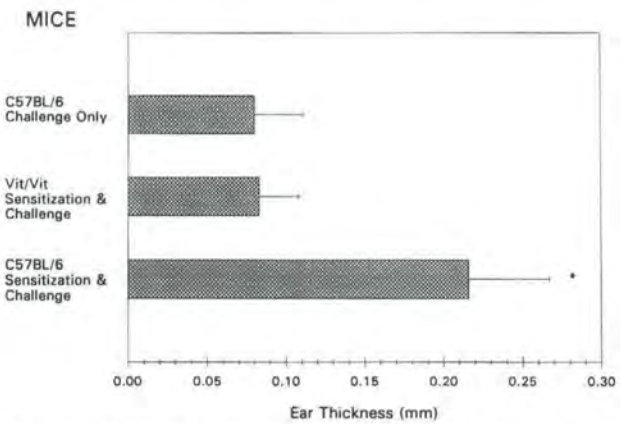

Figure 1. Contact sensitization response of C57BL/6 and mi^{vit}/mi^{vit} mice to TNCB. Four of 6 animals have been sensitized with the hapten (100 µl to 7% TNCB) on abdominal skin and 6 days later challenged on the ears with 20 µl of 1% TNCB. The increment of ear thickness was evaluated 24 hours after elicitation. Bars represent mean+S.D. * Significantly different from the response of similarly treated mi^{vit}/mi^{vit} mice ($p<0.0005$).

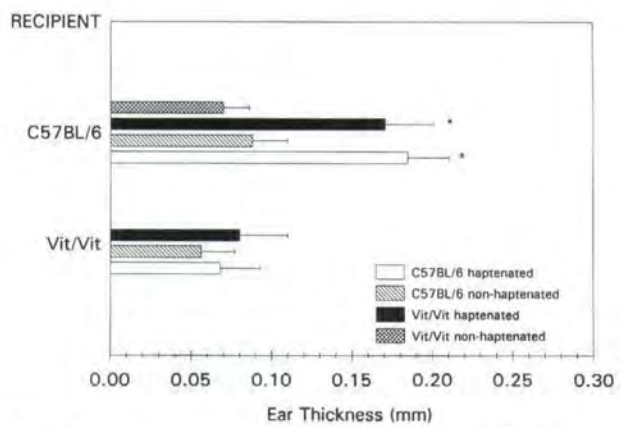

Figure 2. Contact sensitization responses of C57BL/6 and mi^{vit}/mi^{vit} mice sensitized by subcutaneous injection of 10^6 hapten (TNBS)-conjugated or non-haptenized epidermal cells of either C57BL/6 or mi^{vit}/mi^{vit} origin. The animals were challenged on the ears with TNCB (20 µl of 1% solution) 6 days after the subcutaneous injection and the increment of ear thickness was evaluated 24 hours after elicitation. Bars represent mean+S.D. Four to 6 animals were used in each experimental group. * Significantly different from the response of similarly treated mi^{vit}/mi^{vit} or control treated C57b2/6 mice ($p<0.0005$).

contact sensitivity responsiveness to the allergen TNCB in fully depigmented 6- to 8-month-old mi^{vit}/mi^{vit} mice. The animals mounted hardly any challenge response when sensitization was done by painting the skin with the allergen (Fig. 1). To determine if the animals could be sensitized by other routes, other experiments were performed. 10^6 hapten-conjugated viable epidermal cells from mi^{vit}/mi^{vit} or C57BL/6 mice were injected subcutaneously into naive mi^{vit}/mi^{vit} or into naive C57BL/6 recipients. The animals were challenged on the ears 6 d later. Subcutaneous injection of hapten-conjugated epidermal cells from either C57BL/6 or from mi^{vit}/mi^{vit} mutants induced vigorous sensitization of theC57BL/6 recipient (Fig. 2). These findings indicate that the mi^{vit}/mi^{vit} epidermal cells are capable of processing and presenting the hapten TNBC when they are conjugated *in vitro* and injected subcutaneously into congenic C57BL/6 mice. In contrast, subcutaneous injection of hapten-conjugated mi^{vit}/mi^{vit} or normal C57BL/6 epidermal cells into mi^{vit}/mi^{vit} mice did not lead to sensitization as measured by the ear swelling response (Fig. 2). These results indicate that cutaneous immune cells, such as Langerhans cells or lymphocytes, can function normally within the normal environment of congenic C57BL/6 mice. In contrast, C57BL/6 cutaneous immune cells do not function normally on the defective environment of the skin of mi^{vit}/mi^{vit} mice.

Adoptive transfer of lymphocytes

To study whether lymphocytes could respond to allergen *in vivo*, adoptive transfer experiments were carried out as described in Methods. Adoptive transfer of spleen cells from C57BL/6 animals which were sensitized with TNCB on their skin 6 d prior to preparation of spleen cell suspension produced a positive challenge response within naive C57BL/6 recipients. Transfer of spleen cells from mi^{vit}/mi^{vit} animals sensitized by TNCB application to skin did not sensitize naive C57BL/6 recipients (Fig. 3). These latter mice could be sensitized by subsequent epicutaneous application of DNFB or TNCB, a response showing they had not been made tolerant. These results indicate that epicutaneous application of DNFB or TNCB is a null event in the mi^{vit}/mi^{vit} animals.

We observed very different results when mice were exposed to allergens by subcutaneous injection. Groups of C57BL/6 or mi^{vit}/mi^{vit} mice were exposed to allergen by intracutaneous injection of TNBS-conjugated epidermal cells from C57BL/6 mice. To document sensitization, some animals were challenged 6 d later by application of TNCB to the ears. Only the C57BL/6 mice responded.

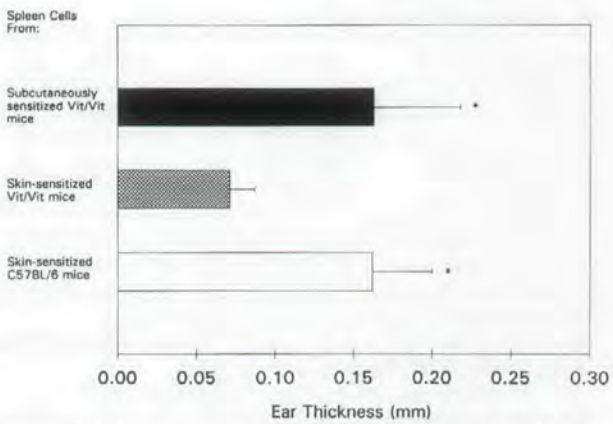

Figure 3. Adoptive transfer of splenic lymphocytes into C57BL/6 mice. 2×10^7 splenic lymphocytes from mice sensitized previously on the skin with 100 µl of 7% TNCB or subcutaneously with TNBS-coupled mi^{vit}/mi^{vit} epidermal cells were injected i.v. into naive C57BL/6 mice. Immediately after transfer the recipients had been challenged with 20 µl of 1% TNCB on the ears and the increment of ear thickness by 24 hours after challenge was evaluated. Bars represent mean+S.D. Four to 6 mice were used in each group. * Significantly different from the response of recipients obtaining transfer from skin-sensitized mi^{vit}/mi^{vit} mice ($p<0.0005$).

The mi^{vit}/mi^{vit} mice showed no inflammatory response by ear swelling.

Other animals of both types from these same groups of mice were sacrificed and splenic lymphocytes prepared as described in Methods. Naive C57BL/6 mice receiving intravenous splenocytes from either C57BL/6 or mi^{vit}/mi^{vit} mice did respond to challenge of TNCB applied to the ear 24 h later (Fig. 3). These results indicate that subcutaneous exposure of mi^{vit}/mi^{vit} mice to allergen sensitizes splenic T lymphocytes, i.e., the lymphocytes are capable of responding to allergen in the proper environment. In addition, the sequence indicates that antigen-presenting cells also function in sites other than the epidermis. We postulated that mi^{vit}/mi^{vit} mice have some abnormality in the epidermal environment that mutes their ability to respond to TNCB or DNFB.

Studies on ICAM-1

Intercellular adhesion molecule-1 (ICAM-1), a ligand for lymphocyte function-associated antigen-1 (LFA-1) (10), is one of the best characterized adhesion/traffic signals of the epidermis. Frozen sections of full thickness ear biopsies obtained from both types of mice sensitized and challenged to allergen were stained using the rat monoclonal antibody YN1/1 which recognizes the murine counterpart of human ICAM-1 (7, 8, 9). The basilar epidermis of the C57BL/6 mice displayed a strong, continuous staining with the antibody. The

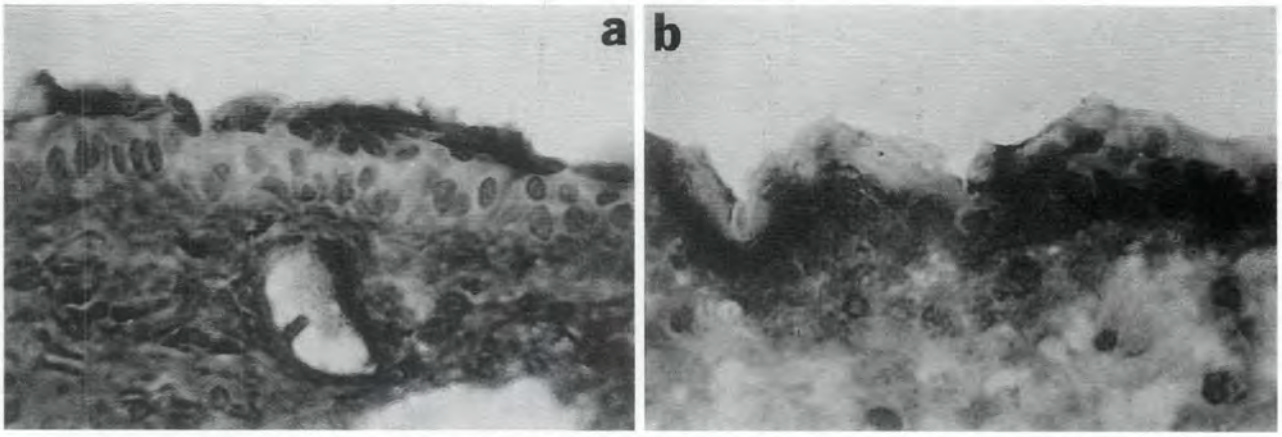

Figure 4. Immunoperoxidase staining of ear biopsies from sensitized mice obtained 48 hours after challenge, with antibody reactive with murine ICAM-1. Note the strong continuous staining of the basilar epidermis of C57BL/6 mice (4A). There is minimal staining in the mi^{vit}/mi^{vit} epidermis (4B) under similar treatment conditions. (Original magnification ×320, counterstain is with 1% toluidine blue-azur-borax).

mi^{vit}/mi^{vit} epidermis, however, essentially did not stain with the antibody under similar treatment conditions. These results suggest a deficiency in expression of ICAM-1 in the mi^{vit}/mi^{vit} epidermis after sensitization and challenge (Fig. 4A and B).

To confirm this, epidermal suspensions from both species of age-matched groups were studied by immunocytochemistry for ICAM-1 expression. Cultured epidermal cells were treated with 50 ng/ml TPA or 400 U/ml recombinant murine IFN-γ for 24 h to induce ICAM-1 expression. The cell yield, density and viability during induction and staining were the same in C57BL/6 and mi^{vit}/mi^{vit} epidermal cultures. The C57BL/6 epidermal cells strongly stained with anti-murine ICAM-1 both after TPA and IFN-γ stimulation whereas the mi^{vit}/mi^{vit} epidermal cells demonstrated only faint staining (Fig. 5A, B and 6A, B). Isotype-matched IgG 2b rat antibody and non-induced targets did not show appreciable staining in C57BL/6 or mi^{vit}/mi^{vit} epidermal cell cultures (not shown).

Flow cytometry

Expression of ICAM-1 by the mi^{vit}/mi^{vit} epidermal cells was considerably lower than by C57BL/6 epi-

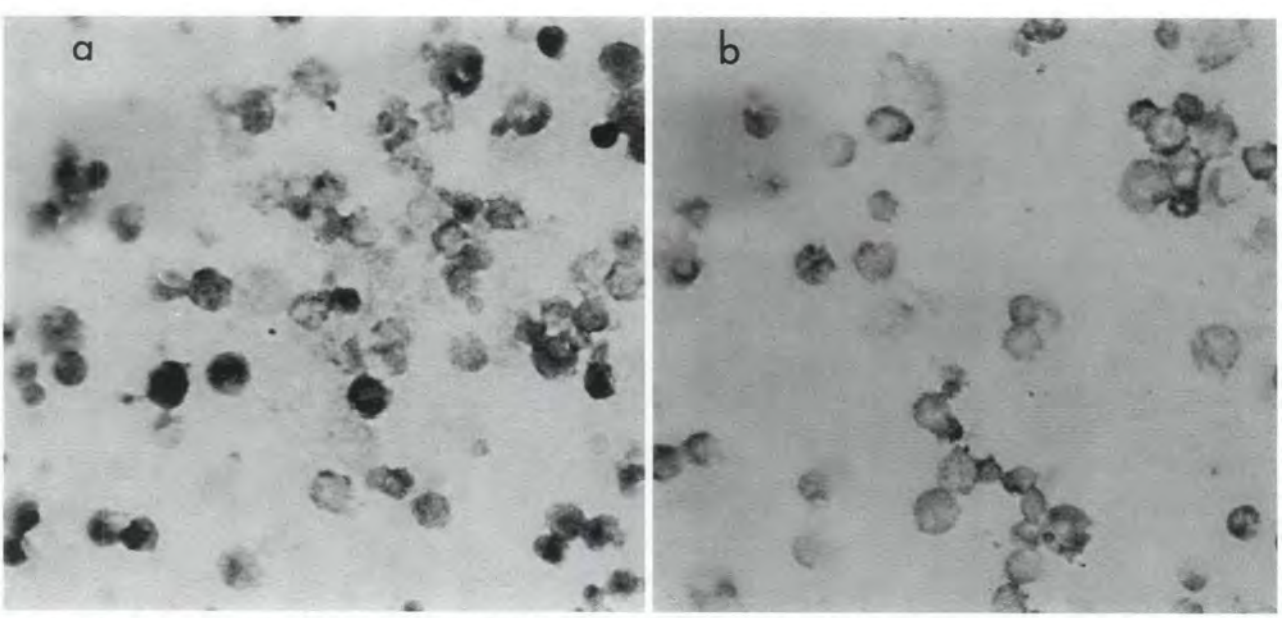

Figure 5. Immunoperoxidase staining of suspensions of murine epidermal cells with antibody reactive with murine ICAM-1. Epidermal cells were stimulated with 50 ng/ml TPA for 24 hours. (A) C57BL/6 epidermal cells; (B) mi^{vit}/mi^{vit} epidermal cells. Note the more intensive staining of C57BL/6 epidermal cells in comparison to the mi^{vit}/mi^{vit} epidermal cells. (Original magnification ×128).

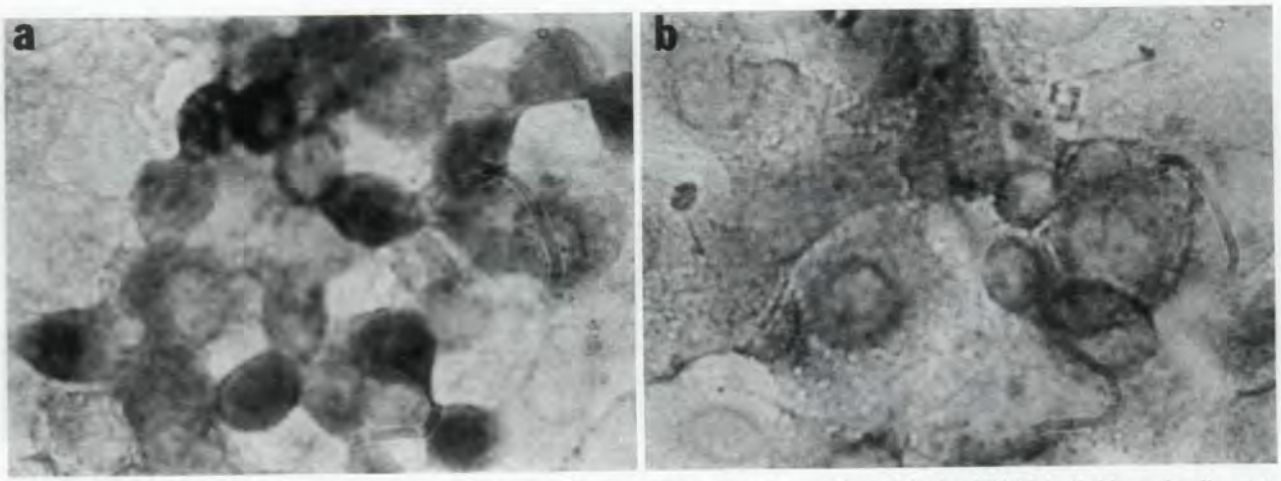

Figure 6. Immunoperoxidase staining of murine epidermal cells with antibody reactive with the ICAM-1. Epidermal cells were stimulated with 400 U/ml IFN-γ for 24 hours. (A) C57BL/6 epidermal cells; (B) mi^{vit}/mi^{vit} epidermal cells. Note the more intensive staining of C57BL/6 epidermal cells in comparison to the mi^{vit}/mi^{vit} epidermal cells. (Original magnification ×168).

dermal cells as measured by flow cytometry as well (Fig. 7). Stimulation with IFN-γ brought about less increment of expression of identifiable ICAM-1 by the mi^{vit}/mi^{vit} epidermal cells than by congenic C57BL/6 epidermal cells. The percentage of mi^{vit}/mi^{vit} and C57BL/6 epidermal cells expressing some ICAM-1 without IFN-γ stimulation was 5.31 and 7.74, respectively. Treatment of cell suspensions with IFN-γ enhanced ICAM-1 staining of mi^{vit}/mi^{vit} and C57BL/6 epidermal cells up to 19.55 and 28.59%, respectively. Because single cell suspensions are not identical to an intact tissue, suspensions were placed in culture for variable time periods in a variety of culture conditions (11) and stimulated by addition of IFN-γ. In some culture conditions with small amounts of IFN-γ added, the difference between the number of mi^{vit}/mi^{vit} cells and normal C57BL/6 cells stained was 29%. This observation seems consistent with staining of tissue (Figs. 4–6) in which ICAM-1 expression is not absent for mi^{vit}/mi^{vit} cells but highly muted. Conditions under which cells were stimulated can significantly alter the response.

Epidermal cell-lymphocyte binding studies

To confirm that expression of ICAM-1 was muted on the mi^{vit}/mi^{vit} cells, functional studies were done. Adhesion of lymphocytes to C57BL/6 or mi^{vit}/mi^{vit} epidermal monolayers was assayed using Con A-stimulated mi^{vit}/mi^{vit} splenic lymphoblasts. Untreated epidermal monolayers from both types of mice bound relatively few of the added lymphocytes (Table 1). Treatment of C57BL/6 epidermal cells with IFN-γ resulted in a significant increase of lymphocyte adhesion. Similar treatment of mi^{vit}/mi^{vit} epidermal cells did not significantly enhance the lymphocyte binding. The addition of YN1/1 antibody partially blocked the lymphocyte binding to stimulated C57BL/6 epidermal cells (Table 1). It had no effect on lymphocyte binding by IFN-γ-treated mi^{vit}/mi^{vit} epidermal cells. Isotype-matched control IgG 2b antibody did not affect lymphocyte binding. These findings provide functional evidence for a deficient epidermal cell/lymphocyte adhesion in the mi^{vit}/mi^{vit}mice.

Discussion

In the present work, we report our results further characterizing the immune deficiency in the mi^{vit}/mi^{vit} mice. We suggest that the defective ability to mount a normal immune/inflammatory response upon epicutaneous application of several contact allergens such as DNFB or TNCB (1, 2, 3) is related to muted expression of adhesion molecules. We concluded this from the results of the new *in vivo* and *in vitro* studies reported here (12). Intracutaneous injections of haptenized cells from mi^{vit}/mi^{vit} mice sensitized splenic lymphocytes, an effect detectable only by adoptive transfer. A significant difference between topical skin sensitization and subcutaneous injection of hapten-conjugated epidermal cells is that the latter procedure bypasses the environment of the epidermis. The technique permits us to conclude that there is no inherent disorder of antigen presentation by the Langerhans cells. This conclusion is substantiated by the normal response of mi^{vit}/mi^{vit} to oxazalone. In addition, subcutaneous injection of haptenized epidermal cells of either C57BL/6 or mi^{vit}/mi^{vit} origin did sensitize naive C57BL/6 mice, another indication that the antigen processing/presentation functions normally outside the environment of the

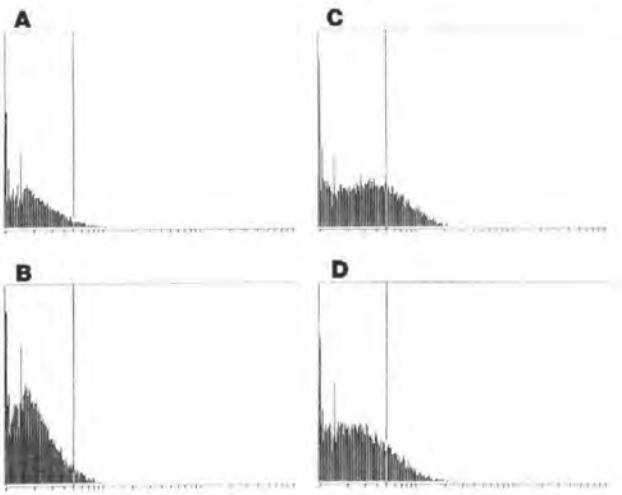

Figure 7. FACS analysis of keratinocyte suspensions before and after incubation in IFN-γ and stained with fluorescein-tagged anti-ICAM antibodies. Fig. 7A shows normal C_{57}BL/6 cells before incubation in interferon. In this run 8% of the normal control cells exhibited a high level of fluorescence. Following incubation in IFN-γ (7C), 29% of the cells exhibited fluorescence above control, and increase of 21%. In contrast, the keratinocytes from the depigmented mice showed 5% fluorescence before exposure to interferon (7B) and 19% after incubation (7D), an increase of 14%. The mean intensity of fluorescence increased from 78 to 89 in the normal cells; but was constant at 77 for the mutant cells. These data confirm that the number of cells and the expression of ICAM was significantly greater in the normal cells stimulated by IFN-γ than in the mutant cells.

epidermis as measured by ear swelling. Mi^{vit}/mi^{vit} mice receiving haptenized epidermal cells either of mi^{vit}/mi^{vit} or C57BL/6 origin did not mount a normal challenge response as measured by ear swelling. Previously we have shown by reciprocal grafting that the mi^{vit}/mi^{vit} mice sensitize through a C57BL/6 graft and respond to challenge with a normal inflammatory reaction (2). These data confirm that mi^{vit}/mi^{vit} lymphocytes can respond to allergens. The splenic lymphocytes of mi^{vit}/mi^{vit} mice are sensitized by intracutaneous injection of hapten-conjugated cells, but such animals do not respond to challenge by ear swelling. Thus antigen presentation can occur within some sites of the mi^{vit}/mi^{vit} mice. It seems that the epidermis is uniquely defective. The defective afferent or efferent response can both be explained by a muted expression of ICAM-1.

That the Langerhans cells are fewer than in C57BL/6 but clearly sufficient in number and function was previously demonstrated by us (2) in experiments which showed that topically applied oxazalone does elicit a vigorous allergic response in mi^{vit}/mi^{vit} mice. This experiment also suggests that Langerhans cells can traffic normally, at least when stimulated by oxazolone.

Spleen cells from mi^{vit}/mi^{vit} mice painted with the hap n on the skin when injected intravenously, did not sensitize naive C57BL/6 recipients. The animals were subsequently sensitized to DNFB. These data confirm that epicutaneous application of allergen to mi^{vit}/mi^{vit} mice is an immunological null event and does not induce tolerance (2). It suggests the epidermis is a poor environment for mounting an immune response. In contrast, mi^{vit}/mi^{vit} epidermal cells contain sufficient numbers of functional antigen-presenting cells to sensitize T cells *in vivo* when the epidermal cells conjugated with the antigen were introduced subcutaneously into C57BL/6 mice. These results suggest that mi^{vit}/mi^{vit} lymphocytes respond to the antigen when introduced in ways that bypass the need for epidermal ICAM expression. The possibility that haptenated antigen-presenting cells, e.g., Langerhans cells from the subcutaneous injection, present in the spleen cell suspension are responsible for the positive adoptive transfer is unlikely. The number of haptenated epidermal cells was 20 times less than the number of spleen cells prepared for the adoptive transfer. In addition, challenge of the recipients was done immediately after the adoptive transfer, which precludes sensitization.

Our studies provide data suggesting that the epidermal environment in mi^{vit}/mi^{vit} mice is not conducive to sensitization with some allergens like DNFB. The Langerhans cells seem to function normally when exposed to oxazalone and when injected subcutaneously into normal mice. The lymphocytes are also capable of responding to DNFB under conditions which bypass the epidermal environment. Our data are most consistent with a defective environment for T lymphocytes or other immunocytes in the epidermis of mi^{vit}/mi^{vit} mice. The subcutaneous injection of hapten-conjugated epidermal cells bypasses the epidermis by T cells or other immunocytes in the afferent sensitization limb. Thus introduction of haptenated cells into the dermis can generate hapten-sensitized T-cell clones in spleens of mi^{vit}/mi^{vit} animals. This conclusion is documented by the positive results of adoptive transfer experiments injecting spleen cells from these animals into naive C57BL/6 mice. The mi^{vit}/mi^{vit} mice themselves, however, remain unable to mount a normal challenge response after subcutaneous sensitization. This again suggests that there is a defect in Langerhans cell-lymphocyte interaction in the epidermis. We observed both histochemically and functionally low epidermal ICAM expression both *in vivo* and *in vitro*. This defect would explain the unique defect in ability to sensitize mice to epidermally applied allergens.

The efferent inflammatory response to challenge also requires expression of ICAM for normal epi-

dermal trafficking of T lymphocytes. The expression of ICAM is muted in these animals, but not absent. Prolonged exposure of skin to IFN-γ does induce a moderate ICAM expression. That ICAM can be expressed in vigorously stimulated cells might explain why mi^{vit}/mi^{vit} mice bearing a C57BL/6 graft can be sensitized and produce a normal elicitation response as we have previously published (1,2).

Frozen sections from full thickness ear biopsies obtained from mice 48 h after challenge with TNCB were studied for ICAM-1 expression (7, 8, 9). Strong staining throughout the basal layer of epidermis was detected on the sections prepared from the C57BL/6 mice. No identifiable staining of the mi^{vit}/mi^{vit} epidermis was seen after hapten challenge.

ICAM-1 is not constitutively expressed in the epidermis and is regulated by inflammatory cytokines such as interferon-γ, tumor necrosis factor-α, and possibly interleukin-1 (13–18). Epidermal cells prepared from C57BL/6 or mi^{vit}/mi^{vit} mice were stimulated with TPA or IFN-γ to enhance ICAM-1 expression. ICAM was expressed on C57BL/6 epidermal cells while the mi^{vit}/mi^{vit} epidermal cells exhibited much weaker reactivity. FACS analysis confirmed the results of immunocytochemistry experiments. From these studies we conclude that the capacity of mi^{vit}/mi^{vit} epidermis to express identifiable ICAM-1 upon stimulation is markedly decreased, although not entirely absent. The histologic data combined with the numerous functional assays strongly suggest that mi^{vit}/mi^{vit} epidermal cells do not express ICAM-1 in a normal manner and might explain the muted responsiveness of these animals to DNFB or TNCB.

ICAM-1 expression by depigmenting epidermis *in vivo* after challenge with the hapten was essentially lacking. Repeated injections of ETAF (epidermal cell-thymocyte activating factor) into the mi^{vit}/mi^{vit} mice increased their contact sensitization response (unpublished) and expression of ICAM. *In vitro*, after stimulation with IFN-γ or TPA, the mi^{vit}/mi^{vit} epidermal cells expressed small quantitites of identifiable ICAM-1, much less than the C57BL/6 epidermal cells. However, prolonged culture of mi^{vit}/mi^{vit} epidermal cells with IFN-γ for 72–96 h produced a moderate expression of ICAM. Because of this observation, we applied DNFB to the skin of mi^{vit}/mi^{vit} mice 5–7 times over periods of 7–9 d. The frequent application of allergen produced two results. There was a strong stain for ICAM-1 and the animals exhibited a normal inflammatory response to challenge with DNFB applied to the ear (data not shown). We conclude that a much more vigorous stimulation of the mi^{vit}/mi^{vit} epidermis is needed to express ICAM-1 in quantities sufficient for a normal contact sensitization response.

These findings provide the first clues to understanding the function of the mi allele on chromosome 6 where the vit mutation in mice is located (19). The mi gene has been cloned and sequenced and is helix-loop-helix protein transcription factor. Several, but not all, proteins secreted by normal C57BL/6 melanocytes are absent in the media of mi^{vit}/mi^{vit} melanocytes maintained in culture. The mi gene seems to affect the transcription of some genes but not all. One of these genes might be the ICAM-1 protein.

Acknowledgments

The authors wish to thank Richard A. Moravec for his excellent technical assistance and Joan Griggs for her secretarial help.

References

1. Rheins L A, Palkowski M R, Nordlund J J. Alterations in cutaneous immune reactivity to dinitrofluorobenzene in graying C57BL/vi · vi mice. J Invest Dermatol 1986: 86: 539–542.
2. Amornsiripanitch S, Barnes L M, Nordlund J J, Trinkle L S, Rheins L A. Immune studies in the depigmenting C57BL/6J-mi^{vit}/mi^{vit} mice. An apparent isolated loss of contact hypersensitivity. J Immunol 1988: 140: 3438–3445.
3. Nordlund J J, Amornsiripanitch S, Rheins L A, Abdel-Malek Z A, Boissy R E, Bell M. Role of the melanocyte in epidermal inflammatory and immune responses. Pigment Cell Res (Suppl) 1988: 1: 101–112.
4. Lerner A B, Shiohara T, Boissy R E, Jacobson K A, Lamoreux M L, Moellmann G E. A mouse model for vitiligo. J Invest Dermatol 1986: 87: 299–304.
5. Uehara M, Miyauchi H, Tanaka S. Diminished contact sensitivity response in vitiliginous skin. Arch Dermatol 1984: 120: 195–198.
6. Hatchome N, Aiba S, Kato T, Torinuki W, Tagami H. Possible functional impairment of Langerhans' cells in vitiliginous skin. Arch Dermatol 1987: 123: 51–54.
7. Horley K J, Carpenito C, Baker B, Takei F. Molecular cloning of murine intercellular adhesion molecule (ICAM-1). EMBO J 1989: 8: 2889–2896.
8. Prieto J, Takei F, Gendelman R, Christenson B, Biberfeld P, Patarroyo M. MALA-2, mouse homologue of human adhesion molecule ICAM-1 (CD54). Eur J Immunol 1989: 19: 1551–1557.
9. Takei F. Inhibition of mixed lymphocyte response by a rat monoclonal antibody to a novel murine lymphocyte activation antigen (MALA-2). J Immunol 1985: 134: 1403–1407.
10. Marlin S D, Springer T A. Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). Cell 1987: 51: 813–819.
11. Abel-Malek Z, Swope V B, Pallas J, Krug K, Nordlund J J. Mitogenic, melanogenic and cAMP responses of cultured neonatal human melanocytes to commonly used mitogens. J Cell Physiol 1992: 150: 416–425.
12. Tamaki K, Fujiwara H, Katz S I. The role of epidermal cells in the induction and suppression of contact sensitivity. J Invest Dermatol 1981: 76: 275–278.

13. Vejlsgaard G L, Ralfkiaer E, Avnstorp C, Czajkowski M, Marlin S D, Rothlein R. Kinetics and characterization of intercellular adhesion molecule-1 (ICAM-1) expression on keratinocytes in various inflammatory skin lesions and malignant cutaneous lymphomas. J Am Acad Dermatol 1989: 20: 782–790.
14. Lewis R E, Buchsbaum M, Whitaker D, Murphy G F. Intercellular adhesion molecule expression in the evolving human cutaneous delayed hypersensitivity reaction. J Invest Dermatol 1989: 93: 672–677.
15. Barker J N W N, Allen M H, MacDonald D M. The effect of *in vivo* interferon-gamma on the distribution of LFA-1 and ICAM-1 in normal human skin. J Invest Dermatol 1989: 93: 439–442.
16. Makgoba M W, Sanders M E, Shaw S. The CD2-LFA-3 and LFA-1-ICAM pathways: relevance to T-cell recognition. Immunol Today 1989: 10: 417–422.
17. Dustin M L, Rothlein R, Bhan A K, Dinarello C A, Springer T A. Induction by IL-1 and interferon-γ: Tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). J Immunol 1986: 137: 245–254.
18. Griffiths C E M, Voorhees J J, Nickoloff B J. Gamma interferon induces different keratinocyte cellular patterns of expression of HLA-DR and DQ and intercellular adhesion molecule-1 (ICAM-1) antigens. Br J Dermatol 1989: 120: 1–7.
19. Lamoreux M L, Boissy R E, Womack J E, Nordlund J J. The *vit* gene maps to the *Mi* (*Microphthalmia*) locus of the laboratory mouse. J Hered 1992: 83: 435–439.