Clin Exp Immunol 1997; **108**:302–308

Calcium channel blockers suppress the contact hypersensitivity reaction (CHR) by inhibiting antigen transport and presentation by epidermal Langerhans cells in mice

N. KATOH, S. HIRANO, S. KISHIMOTO & H. YASUNO *Department of Dermatology, Kyoto Prefectural University of Medicine, Kyoto, Japan*

(Accepted for publication 12 February 1997)

SUMMARY

Since Langerhans cells (LC) are the principal antigen-presenting cells among epidermal cells, treatments suppressing LC function may inhibit CHR. Although calcium channel blockers (CCB) have been shown to suppress the functions of several immunologically active cells, little is known about their effect on LC. In this study we show that pretreatment with topical 1% nifedipine or verapamil HCl significantly suppressed both the sensitization and elicitation phases of a CHR in mice. We then investigated whether CCB affected LC. Flow cytometric analysis of regional lymph node cells obtained 24 h after applying FITC demonstrated that topical CCB treatment significantly reduced the percentage of $FITC^+$ $NLDC\text{-}145^+$ cells, suggesting that CCB had suppressed antigen transport by LC. *In vitro* treatment with nifedipine or verapamil significantly suppressed the antigen-presenting capacity of LC in a dose-dependent manner. In addition, *in vitro* CCB treatment reduced the percentage of class II MHC antigen-positive epidermal cells and significantly suppressed class II MHC and B7-1 levels in LC, as determined by flow cytometry and reverse transcriptase-polymerase chain reaction, whereas surface expression of B7-2 and mRNA was only weakly reduced. Neither expression of CD45 nor the percentage of $CD45^+$ cells were affected, suggesting that the effects of CCB on LC were not due to cytotoxicity. Our results suggest that CCB inhibit CHR, at least in part, by suppressing the functions of LC.

Keywords nifedipine verapamil migration B7-1 Ia

INTRODUCTION

It is now known that the epidermis can serve as the site initiating immune responses, rather than just being a target tissue for these reactions [1]. In CHR, a representative model of a cutaneous immune response, antigen-presenting cells (APC) are essential, since T cells recognize antigenic peptides processed by APC which are combined with class II MHC (Ia) antigens on the cell surface [2,3]. Among epidermal cells (EC), epidermal Langerhans cells (LC) are the principal APC which constitutively express Ia antigens [2,4] and can induce proliferative responses of antigen-primed T cells [5,6]. The function of LC in CHR has already been established. They take up antigen, transport it to primary lymph nodes in the sensitization phase, process it if necessary, and present it to T cells in an Ia-restricted manner [7]. Therefore, it has been suggested that certain treatments which suppress the function of LC could inhibit CHR [8,9].

A number of intracellular events are dependent on calcium ions alone, or calcium in association with its carrier protein calmodulin, as a second messenger. Koide *et al.* investigated the relationship between the level of intracellular calcium and HLA-DR expression, using HL-60 cells which can differentiate into monocytes [10]. They demonstrated that a calcium ionophore A23187-induced calcium influx resulted in increased HLA-DR expression, whereas depleting calcium from the culture medium, or *in vitro* treatment with a calmodulin antagonist, W7, blocked interferon-gamma (IFN-γ)-induced DR expression at the transcription level [10]. These findings imply that calcium might also be involved in the expression of Ia in LC, although the regulation of Ia expression in dendritic cells as LC is likely to differ from expression in other leucocytes [11]. Diezel *et al.* showed that systemic administration of the calcium channel blockers (CCB), lanthanum and diltiazem hydrochloride, inhibited the sensitization phase of hapten-induced CHR in mice, and they suspected that this inhibitory action resulted from the inhibition of LC function [12]. So far, however, they have only demonstrated that CCB diminished LC density and affected the morphology of LC [12]. Although there have also been some reports showing that CCB inhibit cutaneous DTH reactions [13–15], there is little information on the effect of CCB on LC.

In this study, we first investigated whether topically applied CCB could inhibit the sensitization and elicitation phases of hapten-induced CHR using nifedipine and verapamil HCl as the CCB, since systemic CCB treatment might affect not only LC but

Correspondence: Norito Katoh, Department of Dermatology, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kawaramachi Hirokoji, Kamigyo-ku, Kyoto 602, Japan.

also lymphocytes in the lymph nodes [16]. We then examined the effects of CCB on antigen transport, processing, and presentation in order to estimate whether the inhibitory action of CCB in murine CHR is due to the suppression of LC function. We also investigated whether CCB treatment affects the expression of Ia antigens and co-stimulatory molecules, B7-1 and B7-2, which are required for antigen presentation [17].

MATERIALS AND METHODS

Animals

Male BALB/c mice were obtained from Japan SLC (Shizuoka, Japan) and were used at 6–8 weeks of age.

CHR

CHR was assayed by quantifying the ear swelling response to a contact allergen, as previously described [19]. Mice were sensitized by applying 25 μl of 1% 1-chloro-2,4-dinitrobenzene (DNCB; Sigma Chemical Co., St Louis, MO) in a 4:1 acetone–olive oil mixture to the shaved back of the mice. Seven days after sensitization, the mice were challenged with 25 μl of 0·25% DNCB applied to the dorsal surface of the ears. Ear thickness was measured with an engineer's micrometer (Peacock Inc., Tokyo, Japan) at 24 h and 48 h after being challenged and was quantified as the difference in thickness before and after being challenged.

CCB treatment

Nifedipine and verapamil HCl (Sigma) were dissolved and diluted in methyl alcohol at a concentration range 0·01–3% (w/v) and 50 μl of the dilution were applied topically for 2 consecutive days before painting DNCB onto the shaved back and the right ear of the mice to evaluate its effects on sensitization and elicitation, respectively. For the *in vitro* study, nifedipine and verapamil HCl were dissolved in ethyl alcohol at 2×10^{-2} M and diluted serially with medium. The final ethyl alcohol concentration was no greater than 0·5% (v/v) in any working dilution.

EC preparations, culture and enrichment

EC suspensions were prepared from mouse truncal skin treated with Dispase (type II; Godo-Syusei, Tokyo, Japan) as described elsewhere [18]. EC suspensions (1×10^6 cells/ml) were cultured in RPMI 1640 (Nissui Pharmaceutical Co., Tokyo, Japan) containing 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 80 μg/ml kanamycin sulfate, and 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a 5% CO_2 humidified atmosphere. LC enrichment was carried out with Lympholyte M gradients (Cedarlane Labs Ltd, Ontario, Canada) as previously described [20]. LC were enriched to 15–25% by gradient centrifugation and were used in the phenotypic and functional analyses. For RNA preparation, EC were depleted, and enriched for LC by immunomagnetic separation with rat anti-I-$A^{b,d,q}$, I-$E^{d,k}$ MoAb (M5/114.5.2; American Type Culture Collection, Rockville, MD) and sheep anti-rat IgG-coated magnetic beads (Dynabeads M-450; Dynal, Oslo, Norway), as previously described [21]. While 70–80% of the cells in the enriched LC population were Ia-positive, this figure was < 0·2% in the LC-depleted EC population.

Flow cytometry

EC suspensions were cultured in the presence of various concentrations of CCB [16] for 48 h and then enriched for LC. These cells were first incubated with anti-CD32/16 MoAb (2.4G2; Pharmingen, San Diego, CA) for 3 min and then double-stained with FITC-conjugated anti-I-A^d MoAb (AMS–32.1; Pharmingen) and PE-conjugated anti-B7-1 MoAb (1G10; Pharmingen) or PE-conjugated anti-B7-2 MoAb (GL1; Pharmingen), or isotype-matched mouse or rat control antibodies for 30 min on ice. The expression of CD45 on LC was estimated with FITC-conjugated rat anti-mouse pan-CD45 MoAb (M1-9.3HL (Boehringer Mannheim, Indianapolis, IN) and RA3-6B2 (Cedarlane)) in another tube to which propidium iodide (Sigma) was added at a final concentration of 0·5 μg/ml to each sample just before analysis to identify the dead cells. The stained cell populations were analysed with FACScan (Becton Dickinson, San Jose, CA).

Antigen uptake and transport

The capacity of LC to take up and transport antigens to regional lymph nodes was estimated as previously reported [8,9,22]. Mice were pretreated topically on the rear footpads with 1% CCB or vehicle for 2 consecutive days. Then, 25 μl of 0·5% FITC (Sigma) diluted in acetone/dibutylphthalate (1:1) were applied topically to the rear footpads. After 24 h, the draining popliteal lymph nodes were taken out and single-cell suspensions were prepared by pressing the nodes through nylon mesh. These cells were first incubated with rat MoAb NLDC-145 (Serotec, Oxford, UK) [23] for 30 min and then with PE-conjugated anti-rat IgG antibody (Caltag, CA). The mean fluorescence intensities (MFI) and percentages of $FITC^+$ $NLDC\text{-}145^+$ cells were determined with FACScan.

Antigen presentation to presensitized lymphocytes

The ability of LC to present protein antigens was estimated as described previously, with some modifications [9,11]. Antigen-primed lymph node cells were obtained from popliteal and brachial lymph nodes 7 days after priming the footpads of BALB/c mice with 100 μg of keyhole limpet haemocyanin (KLH; Sigma) in 50 μl Freund's complete adjuvant (Sigma). Then the lymph node cells were passed through a Cellect Mouse T-cell Kit (Biotex Labs Inc., Alberta, Canada) according to the manufacturer's protocol and used as KLH-primed T cells. LC-enriched suspensions were adjusted to a concentration of 5×10^6 cells/ml and pulsed with 100 μg KLH for 12 h in the presence of various concentrations of CCB or solvent at 37°C in 5% CO_2. After pulsing, the EC were washed four times in medium, incubated with 50 μg/ml of mitomycin C (Sigma) at 37°C for 30 min, and washed four times. These LC-enriched suspensions (1×10^5/well) were co-cultured with antigen-primed T cells (2×10^5/well) in the absence of additional antigen in 96-well flat-bottomed microtitre plates for 48 h, according to the result of a preliminary study (data not shown). T cell proliferation was estimated by a modification of the MTT assay [24,25] using Cell Titer 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's instructions. The absorbance of the formazan product at 490 nm was measured with a microplate reader (Model 450; BioRad, Hercules, CA). We confirmed in our preliminary study that the data from proliferation bioassays comparing this system and ^{3}H-thymidine incorporation showed similar results.

Reverse transcriptase-polymerase chain reaction

Reverse transcriptase-polymerase chain reaction (RT-PCR) for Ia antigens, B7-1 and B7-2 was carried out using LC-enriched and LC-depleted samples. Total RNA was isolated from these samples after 24 h of culture in the presence or absence of CCB, as

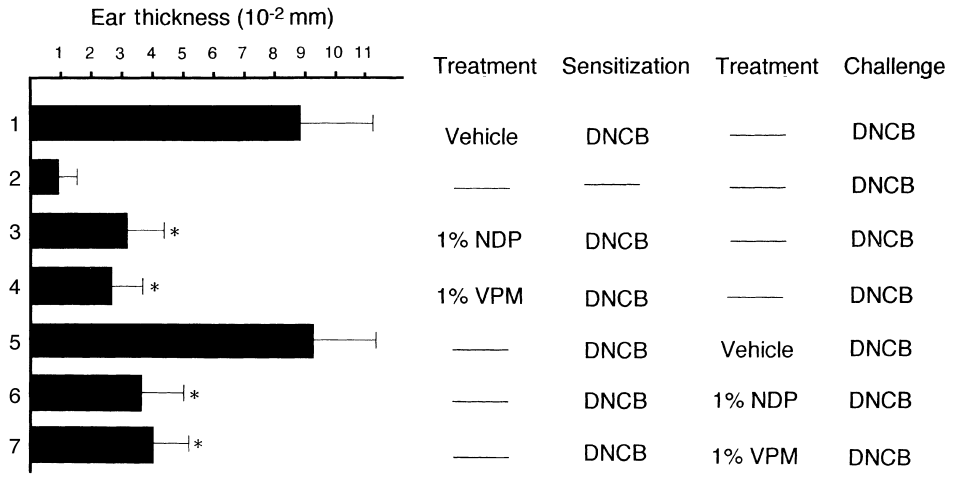

Fig. 1. *In vivo* effects of calcium channel blockers (CCB) on 1-chloro-2,4-dinitrobenzene (DNCB)-induced CHR. Topical treatment with 500 μg of nifedipine (NDP), verapamil hydrochloride (VPM), or vehicle for 2 consecutive days was followed by sensitization or elicitation with DNCB. CHR was estimated by measuring ear swelling. Each measurement is the mean value $\pm$ s.d. ($n = 3$) from a representative experiment. *Significantly different from the mean value of the ear swelling in mice treated with vehicle, $P < 0{\cdot}05$.

described [26]. Total RNA (1 μg) was reverse transcribed using a First-Strand cDNA Synthesis Kit (Pharmacia Biotech, Uppsala, Sweden) in a final reaction volume of 15 μl. The PCR mixture contained 5 μl of the cDNA reaction mixture, 5 μl of 10 $\times$ PCR buffer (Perkin Elmer, Irvine, CA), 0·4 mM dNTPs, 20 pmol of 5′ and 3′ oligonucleotide primers for I-Aβ (a kind gift of Dr Y. Koyama, Nippi Research Institute of Biomatrix; sense: nucleotides 24–42; antisense: nucleotides 264–285), murine B7-1 [27], murine B7-2 (designed on the basis of the published sequence for murine B7-2 [28]; sense: nucleotides 411–432; anti-sense: nucleotides 892–913) or glyceraldehyde 3-phosphate dehydrogenase (G3PDH; Clontech, Palo Alto, CA), and 2·5 U Taq polymerase (Perkin Elmer). Aliquots were then amplified by 30 cycles in a thermal cycler (PHC-1; Techne, Princeton, NJ). Each cycle consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1·5 min, and polymerization at 72°C for 2 min. The amplified PCR products (5 μl) were then electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. The negative films were scanned with a photoscanner (Canon, Tokyo, Japan) and analysed with NIH Image software. Specificity of the amplified bands was validated by their predicted size (262 bp, 483 bp, 503 bp and 983 bp for I-Aβ, B7-1, B7-2 and G3PDH, respectively).

Statistical analysis

Student's *t*-test was used to compare results. $P < 0{\cdot}05$ was considered significant.

RESULTS

Reduction of DNCB-induced ear swelling

Topical application of 1–3% nifedipine or verapamil diluted in methyl alcohol on 2 consecutive days before sensitization significantly reduced DNCB-induced ear swelling (Fig. 1a). The same treatments before elicitation also significantly suppressed the DNCB-induced ear swelling response (Fig. 1b).

Antigen transport by LC

LC are thought to migrate from the epidermis into regional lymph nodes after antigen uptake, which is followed by the presentation of antigen to responsive T cells in the sensitization phase of CHR [29]. After painting of FITC on the skin as hapten, FITC-labelled dendritic cells appeared in the draining lymph nodes [22]. Therefore, we analysed by flow cytometry whether or not CCB affect the ability of LC to take up and transport FITC from the footpads to the popliteal lymph nodes. As seen in Table 1, topical treatment with 1% nifedipine or verapamil on the footpads before FITC painting significantly reduced the percentage of $FITC^+$ $NLDC\text{-}145^+$ cells in the popliteal lymph nodes of CCB-treated mice compared with vehicle-treated mice. The MFI of FITC induced by $NLDC\text{-}145^+$ cells also diminished significantly in lymph node cells of CCB-treated mice.

Antigen presentation by LC

We next investigated whether CCB could affect the antigen-presenting capacity of LC which was estimated from the KLH-primed T cell proliferation reaction. In each experiment, the

Table 1. Antigen transport to the draining lymph nodes after *in vivo* calcium channel blocker (CCB) treatment

Treatment	$FITC^+NLDC\text{-}145^+$ cells (%)	MFI†
0·5% FITC	2·43 $\pm$ 0·32	656·6 $\pm$ 30·2
1% nifedipine + 1% FITC	1·48 $\pm$ 0·16*	341·8 $\pm$ 32·1*
1% verapamil + 1% FITC	1·32 $\pm$ 0·14*	364·4 $\pm$ 42·7*
Vehicle	0·21 $\pm$ 0·03	266·6 $\pm$ 17·8

Topical treatment with 200 μg CCB for 2 days was followed by administration of 0·5% FITC 25 μl on each footpad. After 24 h, the popliteal lymph nodes were removed. The cell suspensions were prepared, immunostained with NLDC-145 and then analysed with FACScan. These data represent three separate experiments and are expressed as mean values $\pm$ s.d. ($n = 3$). * Significantly different from the mean value of mice treated only with FITC, $P < 0{\cdot}05$.

†MFI, Mean fluorescence intensities were determined from the computer-generated log fluorescence histograms. $FITC^+NLDC\text{-}145^+$ cells and the calculated linear fluorescence units are presented.

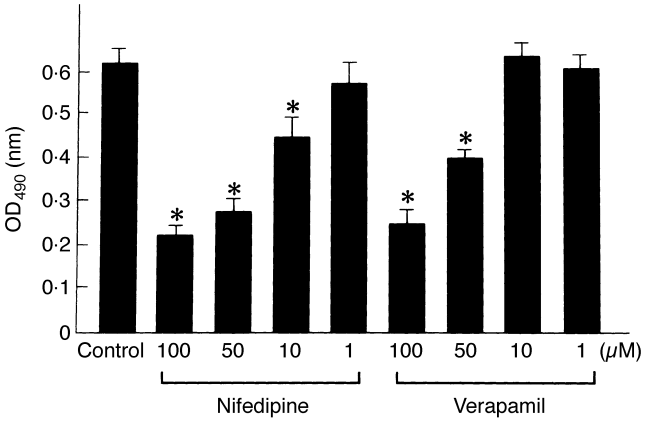

Fig. 2. The effect of calcium channel blockers (CCB) on the antigen-presenting capacity of Langerhans cells (LC), estimated by the keyhole limpet haemocyanin (KLH)-specific T cell response. These data are representative of four separate experiments and are expressed as the mean absorbance value at 490 nm ± s.d. of triplicate cultures. (Mean 'background' (not antigen-pulsed) absorbance value was subtracted from the absorbance value of antigen-pulsed wells.) *Significantly different from the mean value of cultures in the absence of CCB, $P < 0{\cdot}05$.

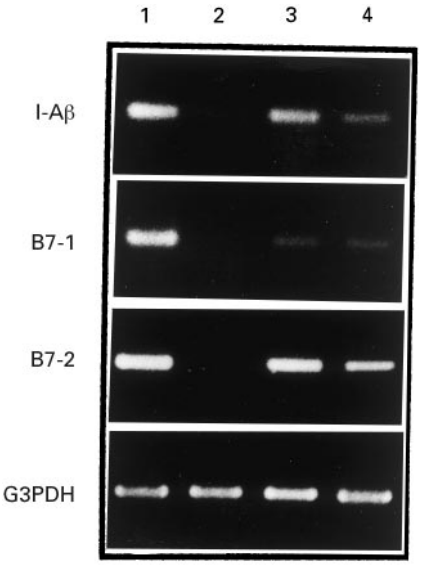

Fig. 3. *In vitro* treatment with calcium channel blockers (CCB) down-regulates the level of I-Aβ and B7-1 mRNA. Langerhans cell (LC)-enriched suspensions (1×10^6/ml) were cultured in the absence (lane 1) or presence of 100 μM nifedipine (lane 3) or 100 μM verapamil hydrochloride (lane 4). LC-depleted suspensions were also cultured (lane 2). RNA was extracted 24 h later, as described, and analysed by reverse transcriptase-polymerase chain reaction.

absorbance value was proportional to the number of living cells using murine lymphocytes. As shown in Fig. 2, *in vitro* presentation of KLH by LC was significantly inhibited with nifedipine and verapamil in a dose-dependent manner at concentrations ranging from 10 to 100 μM and 50 to 100 μM, respectively. Very similar dose-dependent suppression of the antigen-presenting capacity was observed with CCB treatment *in vitro* as determined in allogeneic mixed epidermal cell–lymphocyte reactions using spleen T cells of C3H/HeN mice (data not shown).

Ia antigens, B7-1 and B7-2 expression in LC

It is well known that both Ia antigens and B7 play crucial roles in the antigen presentation of the CHR [3,17]. The levels of Ia, B7-1 and B7-2 in LC are higher after short-term culture than in freshly prepared LC [30–33]. We therefore investigated the ability of CCB to inhibit their expression in short-term cultured LC. In addition to this, we examined pan-CD45 expression in EC, which does not change during culture [33]. As shown in Table 2, 50–100 μM nifedipine or 100 μM verapamil significantly reduced the percentage of Ia-positive cells. Also, both CCB suppressed the expression of the Ia antigen on a per cell basis at a concentration of 100 μM, as determined from the MFI. In addition, 50–100 μM of nifedipine and verapamil significantly reduced the levels of B7-1 expression,

Table 2. Effect of calcium channel blockers (CCB) on Ia and B7 expression in cultured Langerhans cells (LC)

Treatment	Ia-positive cells (%)	Ia expression (MFI†)	$CD45^+$ cells (%)	B7-1 expression (MFI†)	B7-2 expression (MFI†)	Viability (%)
Control	20·1 ± 0·6	2121 ± 77	48·9 ± 5·2	2636 ± 78	9552 ± 282	48·5
Nifedipine						
100 μM	12·0 ± 0·6*	1561 ± 53*	52·3 ± 4·6	1575 ± 45*	8908 ± 224*	51·2
50 μM	17·3 ± 0·4*	2023 ± 82	49·5 ± 4·7	2160 ± 58*	9447 ± 328	49·6
10 μM	19·7 ± 1·2	2152 ± 75	51·8 ± 5·3	2586 ± 50	9254 ± 228	50·2
Verapamil						
100 μM	12·5 ± 1·0*	1258 ± 48*	53·1 ± 5·1	1120 ± 31*	8615 ± 215*	47·3
50 μM	20·8 ± 1·9	1982 ± 71	47·5 ± 5·2	1687 ± 42*	9332 ± 354	48·2
10 μM	21·0 ± 1·3	2098 ± 78	49·1 ± 5·0	2765 ± 38	9622 ± 298	51·4

Epidermal cell suspensions were cultured in the presence or absence of various concentrations of CCB for 48 h. Non-adherent cells were collected and the LC were enriched by density gradients. These cells were stained with the appropriate antibodies and analysed by flow cytometry.

* Significantly different from the mean value of the control, $P < 0{\cdot}05$.

† MFI, Mean fluorescence intensities were determined from the computer-generated log fluorescence histograms, and the calculated linear fluorescence units are presented.

whereas these doses of CCB only suppressed B7-2 expression weakly. Change in the percentage of B7-1- and B7-2 -positive cells by CCB treatment depended on the change in the percentage of Ia-positive cells (data not shown). Neither the expression of CD45 nor the percentage of $CD45^+$ cells was altered by the CCB (data not shown), indicating that CCB treatment was not directly cytotoxic. Normal cell viability was demonstrated in all cultures containing the various drugs by trypan blue staining. There were no significant changes in overall numbers of cells present in cultures treated by CCB and the control (data not shown).

Ia antigens, B7-1 and B7-2 mRNA levels in LC

It was shown that short-term culture of LC increases the levels of B7-1 and B7-2 mRNA [32]. LC express Ia mRNA constitutively [34]. Since it is difficult to obtain a large number of highly purified LC, we employed RT-PCR, although it is not a quantitative method in the strict sense, to determine whether CCB suppress their expression in LC at the mRNA level. As shown in Fig. 3, exposure of LC to 100 μM nifedipine or verapamil for 24 h led to a decrease in the 262-bp and 483-bp fragments, which correspond to I-Aβ mRNA and B7-1 mRNA, respectively. However, the expression of B7-2 mRNA was only weakly suppressed by CCB treatment compared with B7-1. CCB treatments caused no significant changes in the amount of the 983-bp fragment, which corresponds to G3PDH mRNA, suggesting that the effects of CCB on LC are specific and not cytotoxic. Depletion of Ia-positive cells by magnetic immuno-adsorption resulted in the loss of mRNA signals for I-Aβ, B7-1 and B7-2, with no appreciable change in G3PDH mRNA levels.

DISCUSSION

CCB, such as nifedipine, verapamil, and diltiazem, promote the relaxation of cardiac and smooth muscle cells by inhibiting calcium influx through calcium channels (slow channels) and calcium release from intracellular stores, and are commonly used as therapeutic agents for cardiovascular disorders [35–38]. In addition to their effects on muscle cells, several *in vitro* studies have shown that CCB suppress the activation of various participants in immune reactions, such as T cells [16,39], mast cells [40] and macrophages [41,42] via the inhibition of calcium influx, suggesting that CCB can act as an immunosuppressant. Several authors have shown an inhibitory effect of CCB on the cutaneous DTH reaction in *in vivo* studies, which suggested it occurred via the suppression of macrophages and T cells [13–15]. Our results showed that topically applied nifedipine or verapamil suppress not only the elicitation but also the sensitization of CHR in mice. In the sensitization phase, LC interact with T cells in the primary lymph nodes, where topically applied CCB do not seem to work. Therefore, this suggests that the inhibitory effects of topical CCB, especially on the sensitization of T cells, are mainly caused by the suppression of LC function.

In CHR, LC first take up antigens by phagocytosis or pynocytosis [7], then they migrate from the epidermis into the regional lymph nodes [22,29]. Our investigation demonstrated that CCB inhibited the capacity of LC to take up and transport antigens to the regional lymph nodes. Neither the mechanism by which antigens stimulate LC migration, nor the intracellular signalling which leads to LC migration are known. Recently, some reports have demonstrated that ligands for Ia antigens, such as anti-Ia MoAb and staphylococcal superantigens, can induce LC migration [43,44]. If direct binding of haptens to cell surface Ia/peptide complexes can induce the signal for initiating migration of LC [45], reduction of surface Ia expression with CCB, as seen in this study, may be one of the modes by which CCB inhibit LC migration. Halliday & Lucas demonstrated that topical application of a protein kinase C (PKC) activator resulted in LC migration, whereas PKC inhibitors blocked hapten-induced LC migration [46]. They concluded that PKC is involved in the signal initiating LC migration [46]. LC express one of the classical PKC isozymes, PKCβ, whose activity is dependent on intracellular calcium concentration [19,47]. In most of the stimulated cells, transient calcium mobilization is followed by prolonged activation of PKC, and these events cause a variety of cellular responses by acting synergistically [47]. For example, calcium mobilization and the subsequent activation of PKC were shown to be important events in monocyte and B cell stimulation via ligation of Ia antigens [48,49]. Therefore, it is possible that CCB down-regulate LC migration by inhibiting transient calcium release from intracellular stores, and/or by interfering with a sustained calcium influx through calcium channels, which is required for the activation of PKC [35,36].

In this study, the presentation of protein antigens by LC, in which processing is required, was inhibited by CCB in a dose-dependent fashion. We also showed that CCB treatment *in vitro* significantly reduced the percentage of Ia-positive cells and down-regulated the expression and transcription of Ia molecules in LC. Activating T cells to proliferation by APC requires interactions between a T cell receptor and an antigen–Ia complex [3]. Several investigators have demonstrated that a reduction in the antigen-presenting capacity of LC is correlated with a reduction in the surface expression of Ia molecules [8,9,11]. In addition, Puré *et al.* have shown that antigen processing by LC requires newly synthesized Ia molecules [11]. The reduction of Ia mRNA by CCB implies suppression of Ia biosynthesis in LC. Therefore, at least in part, the inhibitory effects of CCB on the expression and transcription of Ia molecules in LC lead to a reduction in the antigen-presenting capacity of LC, and thus to their suppressive effects on ear swelling. The detailed signal transduction mechanisms regulating Ia expression and biosynthesis in LC remain unknown. Our results suggest that calcium mobilization plays an important role in the regulation of Ia expression, and this agrees with findings in HL-60 cells [10]. These problems should be clarified in the near future by studies using recently established long-term LC lines [50] or transgenic mice [51].

Activation of T cells by APC requires another signal or 'co-stimulatory signal' provided by several receptor–ligand interactions, such as CD28 and its counter-receptors, B7-1 and B7-2 [17]. We demonstrated that *in vitro* CCB treatment suppressed both the surface expression and mRNA levels of B7-1. Yokozeki *et al.* reported that allogeneic mixed epidermal cell–lymphocyte reactions were partially inhibited with anti-B7-1 MoAb [32]. Gimmi and co-workers also reported that the antigen-presenting capacity of murine B cells to interact with tetanus toxoid-specific T cells was almost completely blocked with anti-B7-1 antibodies [52]. These facts suggest that a reduction of B7-1 expression on LC, induced by CCB treatment, could account for part of the observed suppressive effects of CCB on antigen presentation, and thus on CHR. Interestingly, in our protocol, CCB treatment suppressed expression of B7-2 to a lesser degree than B7-1 in culture. Although several authors reported that the expression of B7-1 and B7-2 was differentially regulated by cytokine stimulation, the intracellular signals by which their expression is regulated remain

unclear [53,54]. Our results suggest that calcium signals may be more involved in the expression of B7-1 than in the expression of B7-2.

In this study, similar dose–response relationships were observed *in vivo* and *in vitro* for both of the drugs studied. There have been several reports showing that similar concentrations of verapamil and nifedipine inhibit calcium uptake and other calcium channel-mediated events, e.g. in smooth muscle cells [55,56]. Our results imply that the inhibitory effect of nifedipine and verapamil on LC are due to calcium channel blockade. Since nifedipine and verapamil *in vivo* have therapeutic ranges of 0·2–0·88 μM and 0·1 μM, respectively [57,58], the clinical dosages systemically administered against cardiovascular disorders are unlikely to inhibit antigen-presenting capacity of LC significantly. However, it would be possible to administer much higher concentrations to the skin tissue via topical application of CCB, and therefore CCB may offer new therapeutic alternatives in skin diseases in which LC are involved.

ACKNOWLEDGMENTS

We wish to thank Dr Kiyofumi Yamanishi for his generous advice and encouragement in this work, and Dr Youhei Koyama for technical suggestions. This work was supported by a grant from the Ministry of Education, Science, Sports, and Culture, Japan.

REFERENCES

1 Stingl G, Hauser C, Tschahler E, Groh V, Wolff K. Immune functions of epidermal cells. In: Norris DA, ed. Immune mechanisms in cutaneous disease. New York: Marcel Dekker Inc., 1989:3–72.
2 Kondo S, Sauder DN. Epidermal cytokines in allergic contact dermatitis. J Am Acad Dermatol 1995; **33**:786–800.
3 Sette A, Buus S, Colon S, Smith JA, Miles C, Grey HM. Structural characteristics of an antigen required for its interaction with Ia and recognition by T cells. Nature 1987; **328**:395–9.
4 Tamaki K, Stingl G, Guillino M, Sachs DH, Katz SI. Ia antigens in mouse skin are predominantly expressed on Langerhans cells. J Immunol 1979; **123**:784–7.
5 Stingl G, Katz SI, Clement L, Green I, Shevach EM. Immunologic functions of Ia-bearing epidermal Langerhans cells. J Immunol 1978; **121**:2005–13.
6 Stingl G, Gazze-Stingl LA, Aberer W, Wolff K. Antigen presentation by murine epidermal Langerhans cells and its alteration by ultraviolet B light. J Immunol 1981; **127**:1707–13.
7 Romani N, Schuler G, Fritsch P. Identification and phenotype of epidermal Langerhans cells. In: Schuler G, ed. Epidermal Langerhans cells. Boca Raton: CRC Press, 1991:52–66.
8 Blaylock BL, Kouchi Y, Comment CE, Pollock PL, Luster MI. Topical application of T-2 toxin inhibits the contact hypersensitivity response in BALB/c mice. J Immunol 1993; **150**:5135–43.
9 Blaylock BL, Kouchi Y, Comment CE, Corsini E, Rosenthal GJ, Luster MI. Pentamidine isethionate reduces Ia expression and antigen presentation by Langerhans cells and inhibits the contact hypersensitivity reaction. J Immunol 1991; **147**:2116–21.
10 Koide Y, Ina Y, Nezu N, Yoshida TO. Calcium influx and the Ca^{2+}–calmodulin complex are involved in interferon-γ-induced expression of HLA class II molecules on HL-60 cells. Proc Natl Acad Sci USA 1988; **85**:3120–4.
11 Puré E, Inaba K, Crowley MT *et al.* Antigen processing by epidermal Langerhans cells correlates with the level of biosynthesis of major histocompatibility complex class II molecules and expression of invariant chain. J Exp Med 1990; **172**:1459–69.
12 Diezel W, Gruner S, Diaz LA, Anhalt GJ. Inhibition of cutaneous contact hypersensitivity by calcium transport inhibitors lanthanum and diltiazem. J Invest Dermatol 1989; **93**:322–6.
13 Derrenne F, Vanhaeverbeek M, Brohée D. Nifedipine-induced hyporeactivity in delayed hypersensitivity skin tests. Int J Immunopharmacol 1987; **9**:741–4.
14 McFadden J, Bacon K, Camp R. Topically applied verapamil hydrochloride inhibits tuberculin-induced delayed-type hypersensitivity reactions in human skin. J Invest Dermatol 1992; **99**:784–6.
15 Corteza Q, Shen S, Revie D, Chretien P. Effects of calcium channel blockers on *in vivo* cellular immunity in mice. Transplantation 1989; **47**:339–42.
16 Birx DL, Berger M, Fleisher TA. The interference of T cell activation by calcium channel blocking agents. J Immunol 1984; **133**:2904–9.
17 Linsley PS, Ledbetter JA. The role of the CD 28 receptor during T cell responses to antigen. Annu Rev Immunol 1993; **11**:191–212.
18 Garrigue JL, Nicolas JF, Fraginals R, Benezra C, Bour H, Schmitt D. Optimization of the mouse ear swelling test for *in vivo* and *in vitro* studies of weak contact sensitizers. Contact Dermatitis 1994; **30**:231–7.
19 Koyama Y, Hachiya T, Hagiwara M *et al.* Expression of protein kinase C isozyme in epidermal Langerhans cells of the mouse. J Invest Dermatol 1990; **94**:677–80.
20 Aiba S, Katz SI. Phenotypic and functional characteristics of *in vivo*-activated Langerhans cells. J Immunol 1990; **145**:2791–6.
21 Becker D, Reske-Kunz AB, Knop J, Reske K. Biochemical properties of MHC class II molecules endogenously synthesized and expressed by mouse Langerhans cells. Eur J Immunol 1991; **21**:1213–20.
22 Macatonia SE, Knight SC, Edwards AJ, Griffiths S, Fryer P. Localization of antigen on lymph node dendritic cells after exposure to the contact sensitizer fluorescein isothiocyanate. Functional and morphological studies. J Exp Med 1987; **166**:1654–67.
23 Kraal G, Breel M, Janse M, Bruin G. 'Langerhans' cells, veiled cells and interdigitating cells in the mouse recognized by a monoclonal antibody. J Exp Med 1986; **163**:981–7.
24 Melamed D, Friedman A. *In vivo* tolerization of Th1 lymphocytes following a single feeding with ovalbumin: anergy in the absence of suppression. Eur J Immunol 1994; **24**:1974–81.
25 Melamed D, Friedman A. Direct evidence for anergy in T lymphocytes tolerized by oral administration of ovalbumin. Eur J Immunol 1993; **23**:935–42.
26 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-PhOH-chloroform extraction. Anal Biochem 1987; **162**:156–9.
27 Lee MG, Borkowski TA, Udey MC. Regulation of expression of B7 by murine Langerhans cells: a direct relationship between B7 mRNA levels and the level of surface expression of B7 by Langerhans cells. J Invest Dermatol 1993; **101**:883–6.
28 Freeman G, Borriello F, Hodes R *et al.* Murine B7-2, an alternative CTLA-4 counter-receptor that costimulates T cell proliferation and interleukin 2 production. J Exp Med 1993; **178**:2185–92.
29 Kripke ML, Munn CG, Jeevan A, Tang J, Bucana C. Evidence that cutaneous antigen-presenting cells migrate to regional lymph nodes during contact sensitization. J Immunol 1990; **145**:2833–8.
30 Schuler G, Steinman RM. Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells *in vitro*. J Exp Med 1985; **161**:526–46.
31 Shimada S, Caughman SR, Sharrow SO, Stephany D, Katz SI. Enhanced antigen-presenting capacity of cultured Langerhans cells is associated with markedly increased expression of Ia antigen. J Immunol 1987; **139**:2551–5.
32 Yokozeki H, Katayama I, Ohki O *et al.* Functional CD86 (B7-2/B70) on cultured human Langerhans cells. J Invest Dermatol 1996; **106**:147–53.
33 Bieber T, Jürgens M, Wollenberg A, Sander E, Hanau D, de la Salle H. Characterization of the protein tyrosine phosphatase CD45 on human epidermal Langerhans cells. Eur J Immunol 1995; **25**:317–21.
34 Enk AH, Katz SI. Early molecular events in the induction phase of contact hypersensitivity. Proc Natl Acad Sci USA 1991; **89**:1398–402.

35 Walus KM, Fondacaro JD, Jacobson ED. Effects of calcium and its antagonists on the canine mesenteric circulation. Circ Res 1981; **48**:692–700.
36 Saida K, van Breemen C. Mechanism of Ca^{++} antagonist-induced vasodilation. Circ Res 1983; **52**:137–42.
37 Braunwald E. Mechanism of action of calcium channel blocking agents. N Engl J Med 1982; **307**:1618–27.
38 Lee KS, Tsein RW. Mechanism of calcium channel blockade by verapamil, D600, diltiazem and nitrendipine in single dialyzed heart cells. Nature 1983; **302**:790–4.
39 Bacon KB, Westwick J, Camp RDR. Potent and specific inhibition of IL-8-, IL-1α- and IL-1β-induced *in vitro* human lymphocyte migration by calcium channel antagonists. Biochem Biophys Res Commun 1989; **165**:349–54.
40 Tanizaki Y, Akagi K, Lee KN, Townley RG. Inhibitory effect of nifedipine and cromolyn sodium on skin reactions and ^{45}Ca uptake and histamine release in rat mast cells induced by various stimulating agents. Int Arch Allergy Appl Immunol 1983; **72**:102–9.
41 Wright B, Zeidman I, Greig R, Poste G. Inhibition of macrophage activation by calcium channel blockers and calmodulin antagonists. Cell Immunol 1985; **95**:46–53.
42 Shen H, Wiederhold MD, Ou DW. The suppression of macrophage secretion by calcium blockers and adenosine. Immunopharmacol Immunotoxicol 1995; **17**:301–9.
43 Pickard S, Shankar G, Burnham K. Langerhans' cell depletion by staphylococcal superantigens. Immunology 1994; **83**:568–72.
44 Yamashita K, Yano A. Migration of murine epidermal Langerhans cells to regional lymphnodes: engagement of major histocompatibility complex class II antigens induces migration of Langerhans cells. Microbiol Immunol 1994; **38**:567–84.
45 Mommaas AM, Mulder AA, Out CJ *et al.* Distribution of HLA class II molecules in epidermal Langerhans cells *in situ.* Eur J Immunol 1995; **25**:520–5.
46 Halliday GM, Lucas AD. Protein kinase C transduces the signal for Langerhans cell migration from the epidermis. Immunology 1993; **79**:621–6.
47 Nishizuka Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 1992; **258**:607–14.
48 Mooney NA, Grillot Courvalin C, Hivroz C, Ju LY, Charron D. Early biochemical events after MHC class II-mediated signaling on human B lymphocytes. J Immunol 1990; **145**:2070–6.
49 Scholl PR, Trede N, Chatila TA, Geha RS. Role of protein tyrosine phosphorylation in monokine induction by the staphylococcal superantigen toxic shock syndrome toxin-1. J Immunol 1992; **148**:2237–41.
50 Xu S, Ariizumi K, Caceres-Dittmar D *et al.* Successive generation of antigen-presenting, dendritic cell lines from murine epidermis. J Immunol 1995; **154**:2697–705.
51 Longley J, Ding TG, Levin D *et al.* Regulation of transgenic class II major histocompatibility genes in murine Langerhans cells. J Invest Dermatol 1995; **104**:329–34.
52 Gimmi CD, Freeman GJ, Gribben JG, Gray G, Nadler LM. Human T-cell clonal anergy is induced by antigen presentation in the absence of B7 costimulation. Proc Natl Acad Sci USA 1993; **90**:6586–90.
53 Buelens C, Willems F, Delvaux A *et al.* Interleukin-10 differentially regulates B7-1 (CD80) and B7-2 (CD86) expression on human peripheral blood dendritic cells. Eur J Immunol 1995; **25**:2668–72.
54 Larsen CP, Ritchie SC, Hendrix R, Linsley PS, Hathcock KS, Hodes RJ, Lowly RP, Pearson TC. Regulation of immunostimulatory function and costimulatory molecules (B7-1 and B7-2) expression on murine dendritic cells. J Immunol 1994; **152**:5208–19.
55 Triggle DJ, Swamy VC. Calcium antagonists: some chemical-pharmacologic aspects. Circ Res 1983; **52** (Suppl. I):17–28.
56 Millard RW, Grupp G, Grupp IL, DiSalvo J, DePover A, Schwarts A. Chronotropic, ionotropic and vasodilator actions of diltiazem, nifedipine, and verapamil: a comparative study of physiological responses and membrane receptor activity. Circ Res 1983; **52** (Suppl. I):29–39.
57 Robertson DRC, Waller DG, Renwick AG, George CF. Age-related changes in the pharmacokinetics and pharmacodymanics of nifedipine. Br J Clin Pharmacol 1988; **25**:297–305.
58 McMillen MA, Tesi RJ, Baumgarten WB, Jaffe BM, Wait RB. Potentiation of cyclosporine by verapamyl *in vitro*. Transplantation 1985; **40**:444–5.