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Involvement of the Cannabinoid CB2 Receptor and Its Endogenous Ligand 2-Arachidonoylglycerol in Oxazolone-Induced Contact Dermatitis in Mice¹

Saori Oka, Junichi Wakui, Shinobu Ikeda, Shin Yanagimoto, Seishi Kishimoto, Maiko Gokoh, Miwako Nasui, and Takayuki Sugiura²

The possible involvement of 2-arachidonoylglycerol (2-AG), an endogenous ligand for the cannabinoid receptors (CB1 and CB2), in contact dermatitis in mouse ear was investigated. We found that the level of 2-AG was markedly elevated in the ear following a challenge with oxazolone in sensitized mice. Of note, the swelling following the challenge was suppressed by either the administration of SR144528, a CB2 receptor antagonist, immediately after sensitization, or the administration of SR144528 upon the challenge. The effect of AM251, a CB1 receptor antagonist, was marginal in either case. It seems apparent, therefore, that the CB2 receptor and its endogenous ligand 2-AG are closely involved in both the sensitization phase and the elicitation phase of oxazolone-induced contact dermatitis. In line with this, we found that Langerhans cells (MHC class II⁺) contain a substantial amount of CB2 receptor mRNA, whereas keratinocytes (MHC class II⁻) do not. We also obtained evidence that the expression of mRNAs for proinflammatory cytokines following a challenge with oxazolone was markedly suppressed by treatment with SR144528. We next examined whether the CB2 receptor and 2-AG participate in chronic contact dermatitis accompanied by the infiltration of tissues by eosinophils. The amount of 2-AG in mouse ear dramatically increased following repeated challenge with oxazolone. Importantly, treatment with SR144528 attenuated both the recruitment of eosinophils and ear swelling in chronic contact dermatitis induced by repeated challenge with oxazolone. These results strongly suggest that the CB2 receptor and 2-AG play important stimulative roles in the sensitization, elicitation, and exacerbation of allergic inflammation. *The Journal of Immunology*, 2006, 177: 8796–8805.

The Δ^9 -tetrahydrocannabinol, a major psychoactive constituent of marijuana, interacts with specific receptors (cannabinoid receptors), thereby eliciting a variety of pharmacological responses in vitro and in vivo (1). Two types of cannabinoid receptors (CB1 and CB2) have been identified to date. The CB1 receptor is expressed abundantly in the nervous system, especially the brain (2, 3). Evidence is gradually accumulating that shows that the CB1 receptor plays an essential role in the attenuation of synaptic transmission (4). In contrast, the CB2 receptor is expressed predominantly in various lymphoid organs, such as the spleen, tonsils, and lymph nodes (3, 5), and is assumed to participate in the regulation of inflammatory reactions and immune responses (6–10), yet the details remain to be determined.

Two types of arachidonic acid-containing molecules, i.e., *N*-arachidonylethanolamine (anandamide) (11) and 2-arachidonoylglycerol (2-AG)³ (12, 13), have been reported as endogenous ligands for the cannabinoid receptors. Notably, anandamide acted as a partial agonist toward the cannabinoid receptors in various assay systems, whereas 2-AG acted as a full agonist in most cases (14,

15). Importantly, the tissue levels of 2-AG are usually several tens to several hundreds of times those of anandamide (14, 15). Moreover, there is mounting evidence that 2-AG can be rapidly formed from arachidonic acid-containing phospholipids upon stimulation (16–19), whereas no selective and efficient synthetic pathway has been reported for the formation of anandamide in mammalian tissues (15). Based on these observations, we proposed that 2-AG, rather than anandamide, is the true natural ligand for the cannabinoid receptors (14, 15, 20, 21).

We then investigated in detail the biological activities of 2-AG, especially those in the immune system. We found that 2-AG induces the following: 1) a Ca²⁺ transient in HL-60 cells (21); 2) the activation of p42/44 MAPK (22); 3) augmented production of chemokines such as IL-8 in HL-60 cells (23); and 4) migration of HL-60 cells that had differentiated into macrophage-like cells (24), human NK cells (25), and eosinophils (26), through CB2 receptor- and Gi/o-dependent mechanisms. Several investigators also demonstrated that 2-AG induced the migration of mouse splenocytes (27), microglia cells (28), and dendritic cells (29). We also obtained evidence that the CB2 receptor and 2-AG play crucial stimulative roles in 12-*O*-tetradecanoylphorbol-13-acetate-induced acute inflammation in mouse ear (30). These results strongly suggest that the CB2 receptor and 2-AG play essential roles in the stimulation of various types of inflammatory reactions and immune responses. In contrast, several investigators reported that the CB2 receptor and 2-AG may play suppressive roles in inflammatory reactions and immune responses (31–34), although the suppressive effects of exogenous 2-AG are assumed to be due to arachidonic acid metabolites derived from 2-AG rather than 2-AG itself in some cases.

In this study, we examined in detail the possible involvement of the CB2 receptor and 2-AG in contact dermatitis induced by a

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³ Abbreviations used in this paper: 2-AG, 2-arachidonoylglycerol; BHT, butylated hydroxytoluene.

single challenge or repeated challenge with oxazolone. We found that the CB2 receptor and 2-AG play important stimulative roles in both the sensitization phase and the elicitation phase of contact dermatitis induced by a single challenge with oxazolone. We also obtained clear evidence that the CB2 receptor and 2-AG are closely involved in the pathogenesis of chronic contact dermatitis induced by repeated challenge with oxazolone.

Materials and Methods

Chemicals

Arachidonic acid, heptadecanoic acid, and essentially fatty acid-free BSA were purchased from Sigma-Aldrich. Oxazolone, 1-anthroyl cyanide, butylated hydroxytoluene (BHT), and hydrocortisone acetate were obtained from Wako Pure Chemical. Quinuclidine was from Molecular Probes. SR144528 was a gift from Sanofi-Synthelabo. AM251 was purchased from Tocris Cookson. The 1,3-benzylideneglycerol was synthesized from glycerol and benzaldehyde. The 2-AG and 2-heptadecanoylglycerol were prepared from 1,3-benzylideneglycerol and arachidonic anhydride or heptadecanoic anhydride, as described earlier (20). *N*-arachidonylethanolamine (anandamide) and *N*-heptadecanylethanolamine were prepared from ethanolamine and arachidonoyl chloride or heptadecanoyl chloride by the method of Devane et al. (11).

Animals

Male ICR mice (body weight, 30 g) were obtained from Sankyo Labo Service. The present studies were conducted in accordance with the Guide for Care and Use of Laboratory Animals adopted by the Committee on Care and Use of Laboratory Animals of Teikyo University and the Japanese Pharmacological Society.

Oxazolone-induced contact dermatitis in mouse ear

Mice were sensitized with oxazolone (500 μ g) dissolved in 100 μ l of ethanol on the shaved abdomen. Five days after this sensitization, the mice were challenged with oxazolone (100 μ g) dissolved in 20 μ l of acetone on the inner and outer surface of the right ear. Control mice received the vehicle (acetone) on their right ear. Ear thickness was measured 24 h after the challenge using a micrometer.

Oxazolone-induced chronic contact dermatitis in mouse ear

Mice were sensitized with oxazolone, as described above. Five days later, the mice were challenged with oxazolone (100 μ g) dissolved in 20 μ l of acetone on the inner and outer surface of the right ear four times every 5 days. Control mice received the vehicle (acetone) on their right ear. Ear thickness was measured 24 h after each challenge with oxazolone using a micrometer.

Lipid extraction from oxazolone-treated mouse ear

Twenty-four hours after the challenge, the mice were anesthetized with diethyl ether and killed by bleeding from the carotid artery. The ears were then dissected and immediately frozen in liquid nitrogen. They were homogenized in a Bligh and Dyer (35) extraction mixture using a Polytron homogenizer (KINEMATICA). Total lipids were extracted by the method of Bligh and Dyer (35). BHT (final concentration, 0.001%, w/v) was added to avoid lipid peroxidation. The amount of lipid phosphorus was determined by the method of Rouser et al. (36).

Estimation of the amounts of individual molecular species of monoacylglycerols and *N*-acylethanolamines in mouse ear

For the analysis of monoacylglycerols, 2-heptadecanoylglycerol (0.1 nmol) was added as an internal standard. Total lipids were fractionated by TLC with development using petroleum ether:diethyl ether:acetic acid (20:80:1, v/v) in a sealed tank containing N_2 gas. The area corresponding to the standard monoacylglycerol was scraped off the TLC plate and extracted from the silica gel by the method of Bligh and Dyer (35). The extraction was conducted in the presence of BHT (0.001%, w/v) in a N_2 gas-sealed tube. The purified monoacylglycerols were converted to their 1-anthroyl derivatives and then analyzed with an HPLC system equipped with a reverse-phase column (CAPCELL PAK C18 SG120, 4.6 \times 250 mm; Shiseido) and a fluorescence detector (excitation at 370 nm; emission at 470 nm). The mobile phase was acetonitrile:2-propanol:water (90:4:6, v/v), and the flow rate was 1.4 ml/min, as previously described (37). For the analysis of *N*-acylethanolamines, *N*-heptadecanylethanolamine (0.1 nmol) was added as an internal stan-

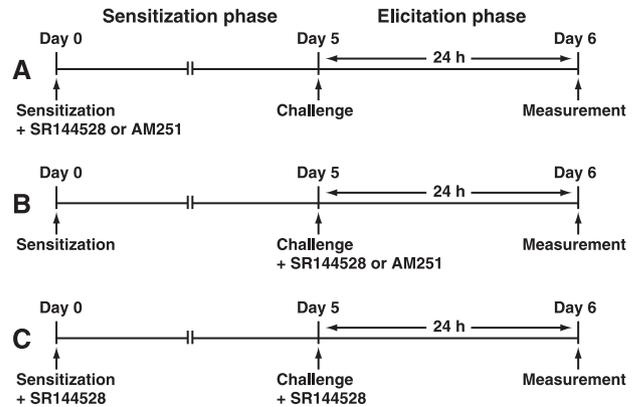


FIGURE 1. Experimental protocols for the evaluation of the effects of cannabinoid receptor antagonists on oxazolone-induced ear swelling. *A*, Mice were sensitized on the abdomen with oxazolone (500 μ g). SR144528 or AM251 (30 μ g each) dissolved in acetone was then applied to the abdomen. Five days later, oxazolone (100 μ g) was applied to the ear of sensitized mice. Twenty-four hours after the challenge, ear thickness was measured using a micrometer. *B*, Mice were sensitized on the abdomen with oxazolone (500 μ g). Five days later, oxazolone (100 μ g) was applied to the ear of sensitized mice. SR144528 or AM251 (30 μ g each) dissolved in acetone was then applied to the same ear. Twenty-four hours after the challenge, ear thickness was measured using a micrometer. *C*, Mice were sensitized on the abdomen with oxazolone (500 μ g). SR144528 (30 μ g) dissolved in acetone was then applied to the abdomen. Five days later, oxazolone (100 μ g) was applied to the ear of sensitized mice. SR144528 (30 μ g) dissolved in acetone was then applied to the same ear. Twenty-four hours after the challenge, ear thickness was measured using a micrometer.

dard. Total lipids were fractionated by TLC with development using chloroform:methanol: NH_4OH (80:20:2, v/v) in a sealed tank containing N_2 gas. The area corresponding to the standard *N*-acylethanolamine was scraped off the TLC plate and extracted from the silica gel by the method of Bligh and Dyer (35). The extraction was conducted in the presence of BHT (0.001%, w/v) in a N_2 gas-sealed tube. The *N*-acylethanolamine was further purified by TLC using first petroleum ether:diethyl ether:acetone:acetic acid (30:40:20:1, v/v) and subsequently an organic layer of ethyl acetate:petroleum ether:diethyl ether:acetic acid:water (100:50:20:100, v/v) as a solvent. *N*-acylethanolamines were converted to their 1-anthroyl derivatives and then analyzed with an HPLC system equipped with a reverse-phase column (CAPCELL PAK C18 SG120, 4.6 \times 250 mm \times 2; Shiseido) and a fluorescence detector (excitation at 370 nm; emission at 470 nm). The mobile phase was acetonitrile:2-propanol:water (80:3:17, v/v), and the flow rate was 1.4 ml/min, as previously described (38).

Effects of cannabinoid receptor antagonists and hydrocortisone acetate on oxazolone-induced ear swelling

SR144528 (a CB2 receptor antagonist) (30 μ g) dissolved in acetone was applied to the abdomen immediately after the sensitization and/or to the ear immediately after the challenge (Fig. 1). In some experiments, various concentrations of SR144528, AM251 (a CB1 receptor antagonist), or hydrocortisone acetate were applied immediately after the challenge with oxazolone. Ear thickness was measured using a micrometer.

Histochemical analysis

The ear was fixed and embedded in paraffin. The block was then cut into 7- μ m sections and stained with H&E. The number of infiltrating cells in the specimen (a cross-section prepared from the central part of the auricle) was estimated by microscopy. To detect the infiltration by eosinophils, specimens were stained with an EoProbe Kit (BioFX Laboratories) and analyzed by fluorescence microscopy.

Preparation of epidermal keratinocytes and Langerhans cells

Naive mouse ear was split into dorsal and ventral halves. Both halves were placed in 0.5% trypsin at 37°C for 1 h. Trypsinized epidermal sheets were peeled from the underlying dermal sheets and filtered through a stainless-steel sieve. The cell suspension was collected and washed twice with Ca^{2+} ,

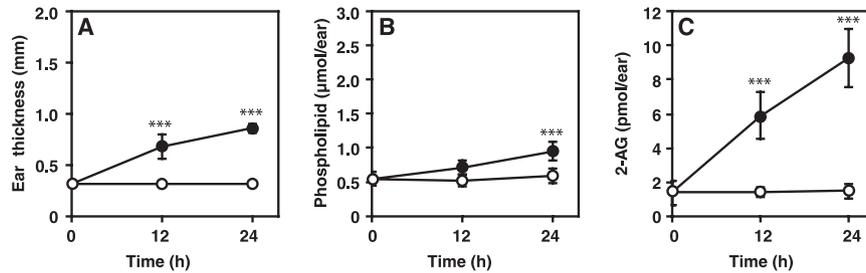


FIGURE 2. The effects of a challenge with oxazolone on thickness, phospholipid content, and the amount of 2-AG in the ear of sensitized mice. The mice were sensitized on the abdomen with oxazolone (500 μg). Five days after the sensitization, the ear was treated with the vehicle (acetone) or oxazolone (100 μg). *A*, The effect of oxazolone treatment on ear thickness was determined using a micrometer, as described in *Materials and Methods*. *B*, The effect of oxazolone treatment on the phospholipid content of mouse ear was determined, as described in *Materials and Methods*. *C*, The effect of oxazolone treatment on the amount of 2-AG in mouse ear was determined using reverse-phase HPLC, as described in *Materials and Methods*. \circ , Control (acetone alone); \bullet , oxazolone. The data are the means \pm SD of five determinations. ***, $p < 0.001$ (compared with control (acetone alone)).

Mg^{2+} -free PBS containing 2% FCS and 0.5% BSA. Cells were incubated with 2 mg/ml mouse IgG at 4°C for 15 min to block Fc receptors. Keratinocytes were obtained by negative selection using MACS CD11c microbeads (Miltenyi Biotec), and Langerhans cells were purified by positive selection using the same separation kit. The purity of keratinocytes and Langerhans cells was assessed by flow cytometry using FITC-conjugated anti-MHC class II (I-A/I-E) Ab (Miltenyi Biotec) and morphologically. The purity of both keratinocytes and Langerhans cells was above 95%.

RT-PCR analysis

Total RNA was extracted from keratinocytes and Langerhans cells using ISOGEN (Nippon Gene). Before reverse transcription, RNA was treated with RNase-free DNase I (Invitrogen Life Technologies), according to the manufacturer's instructions. One microgram of total RNA was then reverse transcribed to cDNA using SuperScript II reverse transcriptase (Invitrogen Life Technologies) with an oligo(dT)₁₈ primer. PCR was conducted using *Ex Taq* polymerase (Takara Bio) under the following conditions: for amplification of the mouse CB2 receptor gene, 30 cycles of 30 s at 94°C, 1 min at 60°C, 1 min at 72°C; for amplification of the GAPDH gene, 25 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C. The following primers were used: CB2 receptor, 5'-TCCTATCATTTACGCCCTGC-3' (sense), 5'-CCTCTGACTCGGGCTGTTTC-3' (antisense); and GAPDH, 5'-ACATCCATGCCATCACTGC-3' (sense), 5'-TCCACCACCTGTTGCTGTA-3' (antisense). Each gene was amplified logarithmically during the number of cycles used in this study. The samples were electrophoresed on a 2% agarose gel. Photographs were taken under UV light.

Quantitative real-time RT-PCR analysis

Total RNA was extracted from mouse ear 24 h after a challenge, and cDNA was synthesized, as described above. Real-time PCR was performed using SYBR Premix *Ex Taq* (Takara Bio) and an Applied Biosystems 7500 real-time PCR system (Applied Biosystems) under the following conditions: 10 s at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C. The following primers were used: MCP-1, 5'-CCAACTCTACTGAAGCCAGCTC-3' (sense), 5'-TTGGACATCTTGTGGTGAA-3' (antisense); MIP-1 α , 5'-TGAACCTACCACTGC-3' (sense), 5'-GGCATTACAGTCCAGGTC-3' (antisense); TNF- α , 5'-AGCACAGAAAGCATGATCCG-3' (sense), 5'-CTTGGTGGTTTGTACGACG-3' (antisense); RANTES, 5'-TCCAACTTGCAGTCGTGTTG-3' (sense), 5'-TCTGGGTTGGCACACACTTG-3' (antisense); eotaxin, 5'-GACCAGTTGGCAAAGAGA-3' (sense), 5'-GGCCTCTGGACCCACTTCT-3' (antisense); eosinophil-associated RNase-1/3/4/12, 5'-TGCTTGAAGTCCCGACTTTGTCT-3' (sense), 5'-GGCATGAGGCAAGCATTAGG-3' (antisense); and GAPDH, 5'-ACATCCATGCCATCACTGC-3' (sense), 5'-TCCACCACCTGTTGCTGTA-3' (antisense). The fold increases in mRNA expression for MCP-1, MIP-1 α , TNF- α , RANTES, eotaxin, and eosinophil-associated RNase-1/3/4/12 over the control levels were calculated after normalization to the GAPDH transcript.

Statistical analysis

Statistical analysis was performed using Student's *t* test (Figs. 3 and 9, B and C) and Tukey's test (Figs. 2, 4–6, 8, and 10). A p value < 0.05 was considered to be significant.

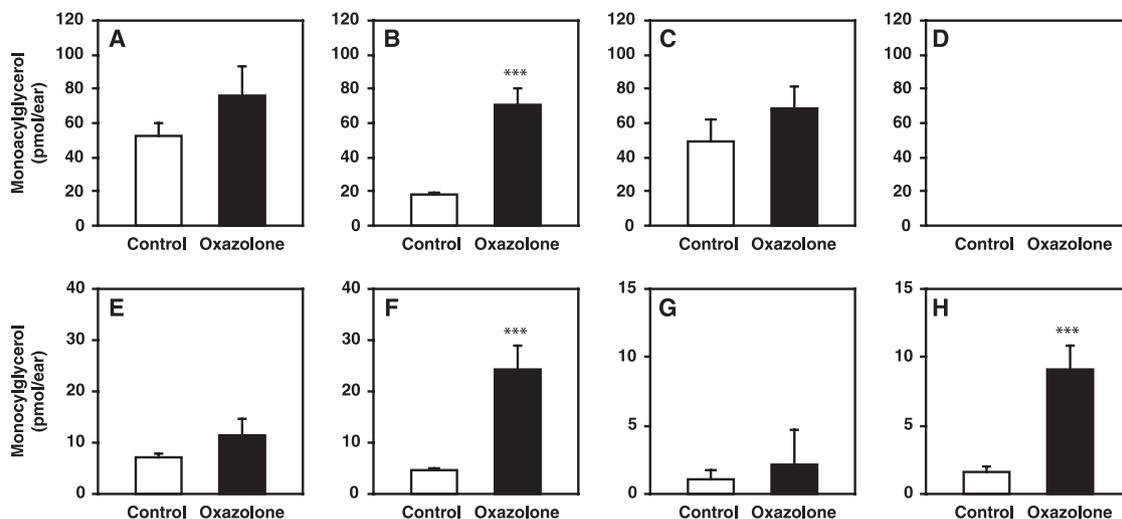


FIGURE 3. The amounts of individual molecular species of monoacylglycerols in mouse ear with oxazolone-induced contact dermatitis. Twenty-four hours after a challenge with oxazolone (100 μg), the amounts of individual molecular species of monoacylglycerols in mouse ear were estimated using reverse-phase HPLC, as described in *Materials and Methods*. *A*, 1(3)-Palmitoylglycerol plus 1(3)-oleoylglycerol plus 1(3)-cis-vaccenoylglycerol; *B*, 2-palmitoylglycerol plus 2-oleoylglycerol plus 2-cis-vaccenoylglycerol; *C*, 1(3)-stearoylglycerol; *D*, 2-stearoylglycerol; *E*, 1(3)-linoleoylglycerol; *F*, 2-linoleoylglycerol; *G*, 1(3)-AG; *H*, 2-AG. The data are the means \pm SD of six determinations. ***, $p < 0.001$ (compared with control (acetone alone)).

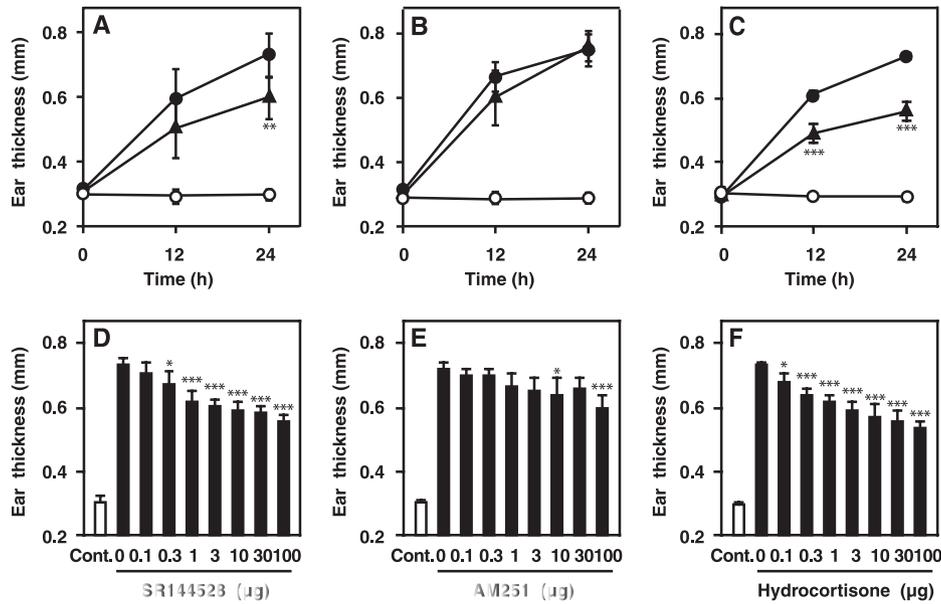


FIGURE 4. The effects of SR144528, AM251, and hydrocortisone acetate applied immediately after sensitization on ear swelling following a challenge with oxazolone in sensitized mice. *A–C*, Mice were sensitized on the abdomen with oxazolone (500 μg). SR144528, AM251, or hydrocortisone acetate (30 μg) dissolved in acetone was then applied to the abdomen. Five days after the sensitization, oxazolone (100 μg) was applied to the ear of sensitized mice. Ear thickness was measured using a micrometer. *A*, SR144528; *B*, AM251; *C*, hydrocortisone acetate. ●, Oxazolone alone; ○, control (acetone alone); ▲, oxazolone plus SR144528 or AM251 or hydrocortisone acetate. The data are the means ± SD of six determinations. **, $p < 0.01$; ***, $p < 0.001$ (compared with oxazolone alone). *D–F*, Mice were sensitized on the abdomen with oxazolone (500 μg). Various concentrations of SR144528, AM251, and hydrocortisone acetate dissolved in acetone were applied onto the abdomen immediately after the sensitization with oxazolone. Five days after the sensitization, oxazolone (100 μg) was applied to the ear of sensitized mice. Twenty-four hours after the challenge with oxazolone, ear thickness was measured using a micrometer. *D*, SR144528; *E*, AM251; *F*, hydrocortisone acetate. The data are the means ± SD of five to six determinations. *, $p < 0.05$; ***, $p < 0.001$ (compared with oxazolone alone).

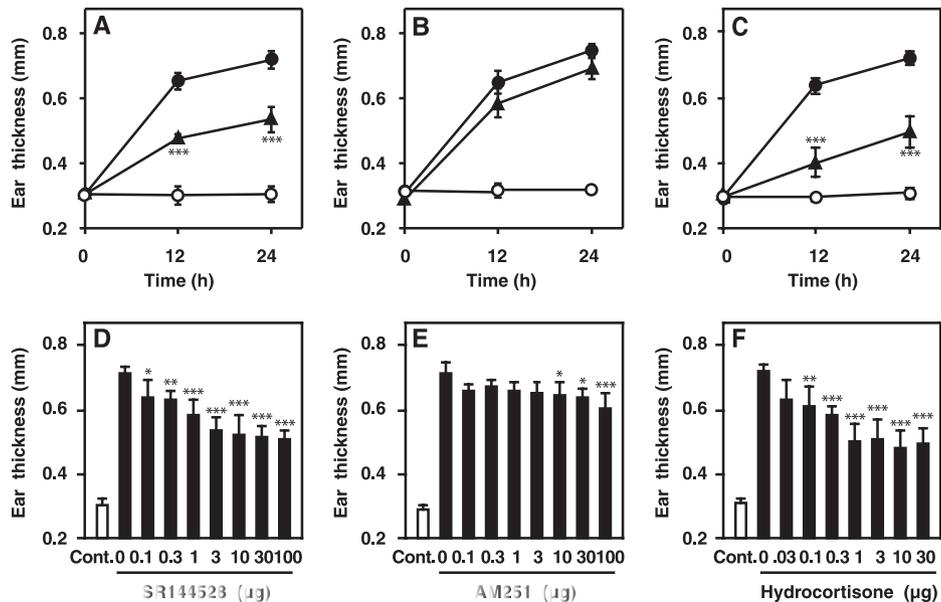


FIGURE 5. The effects of SR144528, AM251, and hydrocortisone acetate applied immediately after the challenge with oxazolone on ear swelling in sensitized mice. *A–C*, Mice were sensitized on the abdomen with oxazolone (500 μg). Five days later, oxazolone (100 μg) was applied to the ear of sensitized mice. SR144528, AM251, or hydrocortisone acetate (30 μg) dissolved in acetone was then applied to the same ear. Ear thickness was measured using a micrometer. *A*, SR144528; *B*, AM251; *C*, hydrocortisone acetate. ●, Oxazolone alone; ○, control (acetone alone); ▲, oxazolone plus SR144528 or AM251 or hydrocortisone acetate. The data are the means ± SD of six determinations. ***, $p < 0.001$ (compared with oxazolone alone). *D–F*, Mice were sensitized on the abdomen with oxazolone (500 μg). Five days later, oxazolone (100 μg) was applied to the ear of sensitized mice. Various concentrations of SR144528, AM251, and hydrocortisone acetate dissolved in acetone were then applied to the same ear. Twenty-four hours after the challenge with oxazolone, ear thickness was measured using a micrometer. *D*, SR144528; *E*, AM251; *F*, hydrocortisone acetate. The data are the means ± SD of five to six determinations. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (compared with oxazolone alone).

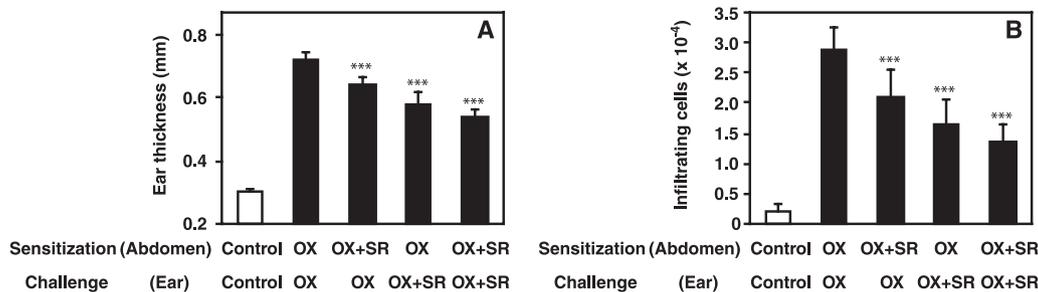


FIGURE 6. The effects of SR144528 on the swelling and the infiltration of tissues by leukocytes in mouse ear with contact dermatitis. *A*, The effects of SR144528 on oxazolone-induced ear swelling. Twenty-four hours after oxazolone (100 μg) was applied to sensitized mice, ear thickness was measured using a micrometer. SR144528 (30 μg) was applied immediately after the sensitization with oxazolone (500 μg) and/or challenge with oxazolone (100 μg). The data are the means \pm SD of eight determinations. ***, $p < 0.001$ (compared with oxazolone alone). *B*, The effects of SR144528 on oxazolone-induced infiltration by leukocytes. The number of infiltrating cells in the specimen (a cross-section prepared from the central part of the auricle) was estimated by microscopy. OX, Oxazolone; SR, SR144528. The data are the means \pm SD of eight determinations. ***, $p < 0.001$ (compared with oxazolone alone).

Results

Effects of the challenge with oxazolone on thickness, phospholipid content, and the amount of 2-AG in the ear of sensitized mice

The challenge with oxazolone elicited contact dermatitis in the sensitized mouse ear. Ear thickness increased by 2.2- and 2.7-fold over the control level at 12 and 24 h, respectively (Fig. 2A). In contrast, the phospholipid content increased only slightly following the challenge with oxazolone (Fig. 2B). We then examined the effect of oxazolone treatment on the amount of 2-AG in mouse ear. As demonstrated in Fig. 2C, the level of 2-AG in the ear was markedly elevated following the challenge. The levels of 2-AG in the ear of oxazolone-treated mice were 1.4 ± 0.7 , 5.9 ± 1.4 , and 9.3 ± 1.7 pmol/ear at 0, 12, and 24 h, respectively.

The amounts of individual molecular species of monoacylglycerols in mouse ear with oxazolone-induced contact dermatitis

We then investigated the effects of the challenge with oxazolone on the amounts of individual molecular species of monoacylglycerols in the ear of sensitized mice. As shown in Fig. 3, vehicle (acetone)-treated control ear contained various molecular species of monoacylglycerols (total 143 pmol/ear). Interestingly, oxazolone treatment induced several-fold increases in the levels of various 2-monoacylglycerols, that is, 2-palmitoylglycerol plus 2-oleoylglycerol plus 2-*cis*-vaccenoylglycerol and 2-linoleoylglycerol, as well as 2-AG (Fig. 3, B, F, and H). In contrast, oxazolone treatment did not markedly affect the levels of 1(3)-monoacylglycerols (Fig. 3, A, C, E, and G).

The amounts of N-acylethanolamines in mouse ear with oxazolone-induced contact dermatitis

We next examined the effects of the challenge with oxazolone on the amounts of *N*-acylethanolamines, including *N*-arachidonoylethanolamine (anandamide), in the ear of sensitized mice. Various species of *N*-acylethanolamines were detected in vehicle-treated control ear (total 270 pmol/ear). In contrast to the case for monoacylglycerols shown in Fig. 3, oxazolone treatment did not markedly influence the levels of any molecular species of *N*-acylethanolamines (data not shown). For instance, the level of anandamide in the ear of control mice was 0.8 ± 0.1 pmol/ear, and that in the ear of oxazolone-treated mice was 1.0 ± 0.1 pmol/ear ($n = 4$).

Effects of cannabinoid receptor antagonists on ear swelling and the infiltration by leukocytes induced by the challenge with oxazolone in sensitized mice

The finding that the endogenous ligand 2-AG was generated concomitant with ear swelling points to the participation of the cannabinoid receptors in contact dermatitis. We then investigated whether cannabinoid receptor antagonists reduce ear swelling elicited by the challenge with oxazolone. We first examined the effects of the application of SR144528, a CB2 receptor antagonist, and AM251, a CB1 receptor antagonist, in the sensitization phase and compared them with that of hydrocortisone acetate. SR144528, AM251, or hydrocortisone acetate (30 μg each) was applied to the abdomen immediately after sensitization with oxazolone on day 0 (Fig. 1A). As shown in Fig. 4A, the application of SR144528 to the abdomen suppressed the ear swelling observed following the challenge with oxazolone on day 6. The application of hydrocortisone acetate also suppressed ear swelling (Fig. 4C). In contrast, AM251 did not exert appreciable effects on ear swelling (Fig. 4B).

Fig. 4, D–F, shows the dose dependency of the effects of SR144528, AM251, and hydrocortisone acetate, applied immediately after sensitization, on ear swelling. SR144528 and hydrocortisone acetate suppressed the swelling in dose-dependent manners. The IC_{50} value of SR144528 was 0.7 μg , and that of hydrocortisone acetate was 0.3 μg (Fig. 4, D and F). In contrast, AM251 did not markedly suppress ear swelling (Fig. 4E). These results suggest that the CB2 receptor, rather than the CB1 receptor, is involved in the sensitization phase of oxazolone-induced contact dermatitis.

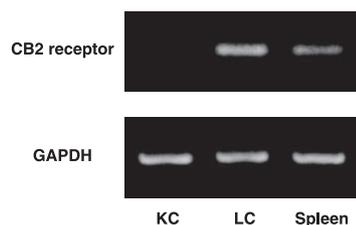


FIGURE 7. Detection of CB2 receptor mRNA in epidermal keratinocytes and Langerhans cells. Keratinocytes and Langerhans cells were prepared from the ears of naive mice. RT-PCR was performed on poly(A)⁺ RNAs, as described in *Materials and Methods*. KC, keratinocytes; LC, Langerhans cells. The results are representative of three separate experiments that gave similar results.

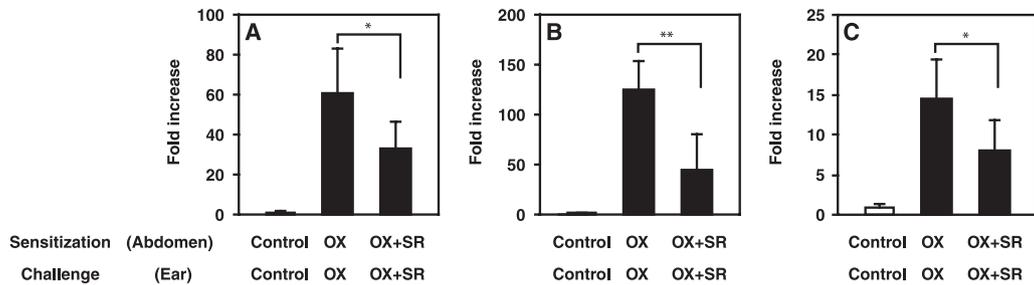


FIGURE 8. The effects of SR144528 on the levels of mRNAs for MCP-1, MIP-1 α , and TNF- α in mouse ear with contact dermatitis. Twenty-four hours after oxazolone (100 μ g) was applied to sensitized mice, the ears were dissected and total RNA was extracted. Quantitative RT-PCR was performed, as described in *Materials and Methods*. A, MCP-1; B, MIP-1 α ; C, TNF- α . SR144528 (30 μ g) was applied upon both sensitization with oxazolone (500 μ g) (abdomen) and challenge with oxazolone (100 μ g) (ear). OX, oxazolone; SR, SR144528. The data are the means \pm SD from four to five determinations. *, $p < 0.05$; **, $p < 0.01$.

The effects of the application of SR144528, AM251, and hydrocortisone acetate in the elicitation phase of contact dermatitis were examined next. SR144528, AM251, or hydrocortisone acetate (30 μ g each) was applied to the ear immediately after the challenge with oxazolone on day 5 (Fig. 1B). As demonstrated in Fig. 5A, the application of SR144528 induced a marked reduction in ear swelling on day 6. The application of hydrocortisone acetate also reduced ear swelling (Fig. 5C). In contrast, only a slight effect was observed with AM251 (Fig. 5B).

Fig. 5, D and E, depicts the dose dependency of the effects of SR144528, AM251, and hydrocortisone acetate, applied immediately after challenge with oxazolone, on ear swelling. SR144528 and hydrocortisone acetate suppressed the swelling dose dependently. The IC₅₀ value of SR144528 was 0.3 μ g, and that of hydrocortisone acetate was 0.1 μ g (Fig. 5, D and F). In contrast, AM251 did not markedly affect ear swelling (Fig. 5E). These results suggest that the CB2 receptor, and not the CB1 receptor, is involved in the elicitation phase, in addition to the sensitization phase, of oxazolone-induced contact dermatitis.

We then investigated in more detail the effect of SR144528 on ear swelling and the infiltration by leukocytes. As demonstrated in Fig. 6A, the application of SR144528 (30 μ g) to the abdomen upon sensitization (Fig. 1A) suppressed ear swelling by 18%. The application of SR144528 (30 μ g) to the ear upon a challenge (Fig. 1B) also reduced ear swelling (33%). Notably, a more pronounced suppression (43%) was observed when SR144528 was applied upon both the sensitization and a challenge (Fig. 1C). Similar results were obtained for the number of infiltrating cells (Fig. 6B).

Comparison of the expression of the CB2 receptor in epidermal keratinocytes and Langerhans cells

The experimental results shown in Figs. 4 and 6 indicate that SR144528 suppressed allergic reaction in the sensitization phase of contact dermatitis. We then examined whether keratinocytes and Langerhans cells, two major cell types of the epidermal layer obtained from naive mice, express CB2 receptor mRNA by RT-PCR. Langerhans cells are immature dendritic cells found in the epidermal layer. They are characterized by the presence of Birbeck granules, the expression of MHC class II Ag, and a dendritic morphology, and play important roles in the induction of sensitization. In contrast to Langerhans cells, keratinocytes do not express the MHC class II Ag. As demonstrated in Fig. 7, Langerhans cells contained a large amount of CB2 receptor mRNA. In contrast, the amount in keratinocytes was negligible. It seems possible, therefore, that the endog-

enously formed 2-AG interacts mainly with Langerhans cells, rather than keratinocytes, to stimulate subsequent immune responses through yet unknown mechanisms.

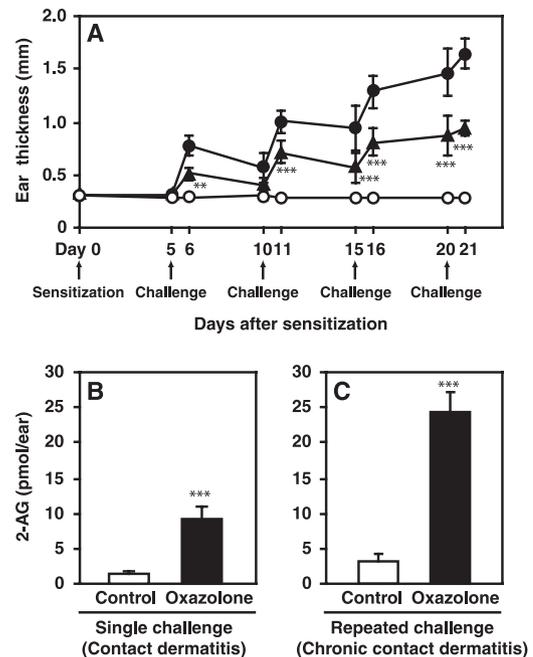


FIGURE 9. The effects of SR144528 on the ear swelling induced by repeated challenge with oxazolone and the amount of 2-AG in the ear with chronic contact dermatitis. A, The effects of SR144528 on ear thickness. Oxazolone (100 μ g) was applied to the ear every 5 days, starting from 5 days after sensitization on the abdomen (500 μ g) (●). SR144528 (30 μ g) dissolved in acetone was applied to the abdomen after sensitization and to the ear after each challenge with oxazolone (▲). Control mice received the vehicle (acetone) instead of oxazolone (○). Ear thickness was measured using a micrometer. The data are the means \pm SD from five determinations. **, $p < 0.01$; ***, $p < 0.001$ (compared with oxazolone alone). B, The amount of 2-AG in mouse ear following a single challenge with oxazolone. Twenty-four hours after the challenge with oxazolone (100 μ g) (day 5), the amount of 2-AG in mouse ear was estimated. Control mice received the vehicle (acetone) on day 5. The data are the means \pm SD of five determinations. ***, $p < 0.001$. C, The amount of 2-AG in mouse ear following repeated challenge with oxazolone. Twenty-four hours after the final challenge with oxazolone (100 μ g) (day 20), the amount of 2-AG in mouse ear was estimated. Control mice received the vehicle (acetone) instead of oxazolone. The data are the means \pm SD of five determinations. ***, $p < 0.001$.

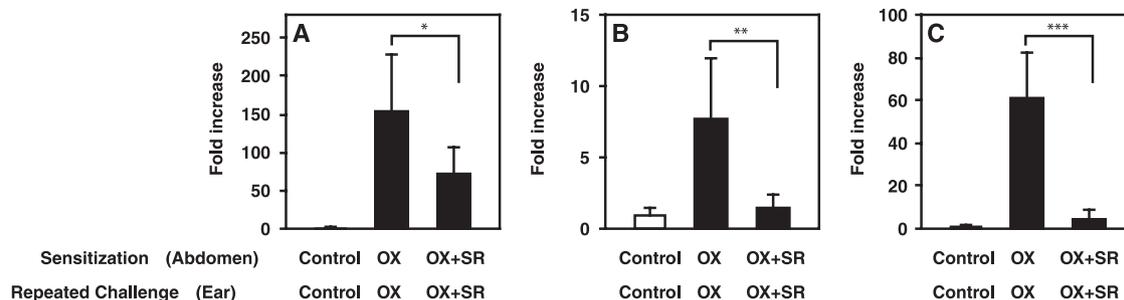


FIGURE 10. The effects of SR144528 on the levels of mRNAs for RANTES, eotaxin, and eosinophil-associated RNase in mouse ear with chronic contact dermatitis. Twenty-four hours after the final challenge with oxazolone (100 μg) (day 20), the ears were dissected and total RNA was extracted. Quantitative RT-PCR was performed, as described in *Materials and Methods*. A, RANTES; B, eotaxin; C, eosinophil-associated RNase. OX, oxazolone; SR, SR144528. The data are the means \pm SD from four to five determinations. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Effects of SR144528 on the expression of mRNAs for inflammatory cytokines in mouse ear with oxazolone-induced contact dermatitis

We then examined the changes in the expression of mRNAs for cytokines in the ear with contact dermatitis. In this experiment, we estimated the levels of the mRNAs for proinflammatory cytokines such as MCP-1, MIP-1 α , and TNF- α using quantitative RT-PCR. The expression of mRNAs for MCP-1, MIP-1 α , and TNF- α increased markedly in the ear challenged with oxazolone (Fig. 8). The effects of SR144528, applied immediately after the sensitization and challenge, were examined next. We found that treatment with SR144528 markedly suppressed mRNA levels of MCP-1, MIP-1 α , and TNF- α (Fig. 8). These results strongly suggest that the endogenous cannabinoid receptor ligand, that is 2-AG, stimulates the production of various proinflammatory cytokines, thereby enhancing allergic inflammation following the challenge with oxazolone.

The effects of SR144528 on ear swelling induced by repeated challenge with oxazolone and the amounts of 2-AG in mouse ear with chronic contact dermatitis

We then examined the possible involvement of the CB2 receptor and 2-AG in chronic contact dermatitis induced by repeated challenge with oxazolone. We applied oxazolone to the mouse

ear every 5 days, starting from 5 days after sensitization on the abdomen. Repeated challenge with oxazolone elicited chronic contact dermatitis in the sensitized mouse ear. As shown in Fig. 9A, repeated application of oxazolone caused dramatic increases in ear thickness. We then examined the effect of SR144528 on oxazolone-induced chronic contact dermatitis. The application of SR144528 (30 μg) to the abdomen after sensitization and to the ear after each challenge markedly suppressed ear swelling at each time point with a suppressive rate of $\sim 50\%$.

The level of 2-AG in the ear with chronic contact dermatitis was examined next. The level was elevated following a single challenge with oxazolone to sensitized mice (Fig. 9B). Importantly, the level of 2-AG dramatically increased after repeated challenge with oxazolone (Fig. 9C).

Effects of SR144528 on eosinophil recruitment in mouse ear with chronic contact dermatitis

We then investigated the levels of mRNA expression of RANTES and eotaxin, which are eosinophil chemotactic proteins, in chronic contact dermatitis. The expression of mRNAs for RANTES and eotaxin increased remarkably in the ear with chronic contact dermatitis induced by repeated challenge with oxazolone (Fig. 10, A

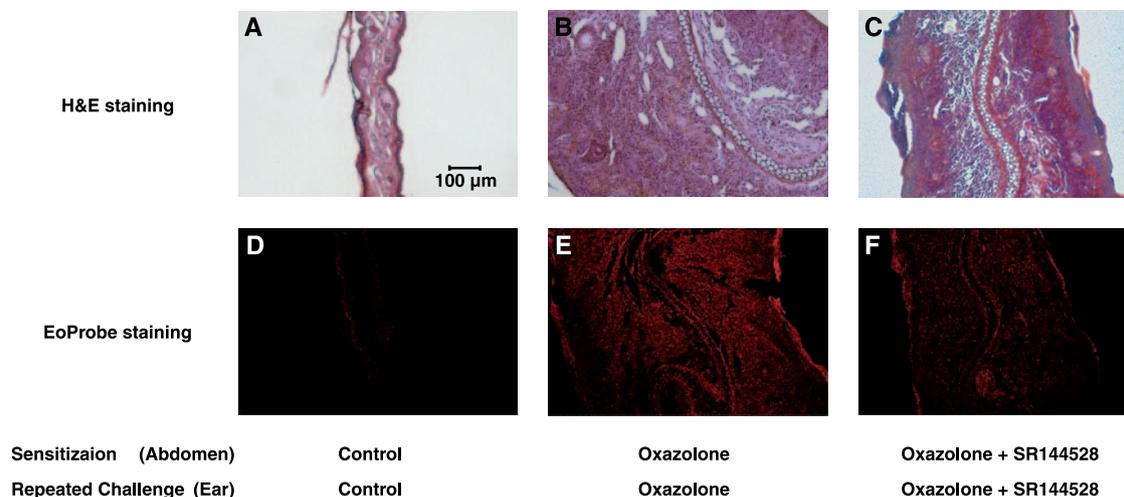


FIGURE 11. The effect of SR144528 on the infiltration of tissues by eosinophils in mouse ear with chronic contact dermatitis. Twenty-four hours after the final challenge with oxazolone (100 μg) (day 20), the ears were dissected and subjected to histochemical analysis, as described in *Materials and Methods*. A and D, Control (acetone alone); B and E, oxazolone; C and F, oxazolone plus SR144528. A–C, H&E staining; D–F, EoProbe staining. SR144528 (30 μg) was applied upon both sensitization with oxazolone (500 μg) (abdomen) and challenge with oxazolone (100 μg) (ear). The results are representative of two separate experiments that gave similar results.

and *B*). Notably, the application of SR144528 (30 μg) after sensitization and each challenge with oxazolone markedly reduced the mRNA expression of RANTES and eotaxin (Fig. 10, *A* and *B*).

We next examined the effect of SR144528 on eosinophil recruitment by measuring mRNA levels of eosinophil-associated RNase, which is located in the granules of eosinophils and has similar physical/functional properties to human eosinophil-associated RNase, eosinophil-derived neurotoxin, and eosinophil cationic protein. As illustrated in Fig. 10C, the application of SR144528 markedly suppressed the increased expression of mRNA for eosinophil-associated RNase induced by repeated application of oxazolone, suggesting that SR144528 suppressed the infiltration of tissues by eosinophils.

This was confirmed by a histochemical analysis. As demonstrated in Fig. 11E, the infiltration by eosinophils occurred in the ear with chronic dermatitis. Importantly, the number of infiltrating eosinophils was markedly reduced in the ear to which SR144528 was applied (Fig. 11F). These results strongly suggest that the CB2 receptor and 2-AG play important roles in the infiltration by eosinophils of tissues with chronic dermatitis.

Discussion

The CB2 receptor is abundantly expressed in several types of inflammatory cells and immune-competent cells, such as B lymphocytes, NK cells, and macrophages/monocytes (3, 5, 39). There is growing evidence that the CB2 receptor plays some essential role in the modulation of several types of inflammatory reactions and immune responses *in vitro* and *in vivo* (6–10, 22–34, 40–42), although the exact physiological and pathophysiological roles of the CB2 receptor have yet to be fully elucidated.

In this study, we examined in detail the pathophysiological roles of the CB2 receptor and its endogenous ligand 2-AG using a mouse model of contact dermatitis. We found that treatment of the ear with SR144528, upon a challenge with oxazolone, markedly reduced ear swelling evoked following the challenge (Figs. 5, *A* and *D*, and 6A). These results strongly suggest that the CB2 receptor plays essential roles in the inflammatory reactions of the elicitation phase of contact dermatitis. We obtained evidence that the amount of 2-AG was markedly increased in the inflamed ear of sensitized mice following the challenge with oxazolone (Figs. 2C and 3H). In contrast to that of 2-AG, the level of anandamide, another endogenous ligand for the cannabinoid receptor, did not change remarkably. It is apparent, therefore, that the relevant endogenous ligand for the CB2 receptor in acute inflammation induced by a challenge with oxazolone is 2-AG, not anandamide. These results are generally the same as those for 12-*O*-tetradecanoylphorbol-13-acetate-induced acute inflammation in mouse ear (30).

The mechanism by which SR144528 suppressed inflammatory reactions following the challenge with oxazolone is not yet fully clear. Notably, the application of SR144528 to the ear of sensitized mice markedly reduced the expression of mRNAs for MCP-1, MIP-1 α , and TNF- α (Fig. 8). This observation is extremely important, because MCP-1, MIP-1 α , and TNF- α are known to play crucial roles in the pathogenesis of contact dermatitis. For example, the ear swelling following a challenge with oxazolone was diminished in TNF- α -deficient mice (43–46), and the recruitment of mononuclear cells was impaired in MCP-1-deficient mice (47). Thus, it seems possible that the CB2 receptor and its endogenous ligand 2-AG participate in the elicitation phase of contact dermatitis by up-regulating the production of these cytokines.

Another important phase of contact dermatitis is the sensitization phase. Notably, the ear swelling observed following the chal-

lenge with oxazolone on day 6 was reduced when SR144528 was applied together with oxazolone upon sensitization on day 0 (Figs. 4, *A* and *D*, and 6A). A similar result was obtained for the number of cells infiltrating the tissues on day 6 (Fig. 6B). These results clearly indicate that SR144528 suppressed the process of sensitization on the abdomen, leading to a reduction in contact hypersensitivity reactions in the ear following the challenge with oxazolone.

The mechanism underlying the SR144528-induced inhibition of the sensitization phase is yet to be determined. Previously, several investigators reported that the CB2 receptor is present in dendritic cells (29, 48). In this study, we obtained evidence that a high level of CB2 receptor mRNA is expressed in Langerhans cells, whereas keratinocytes, which comprise the majority of epidermal cells, did not contain an appreciable amount of CB2 receptor mRNA (Fig. 7). This observation is quite noteworthy, because Langerhans cells are the most important APCs in the epidermis. The exact mechanism through which the CB2 receptor and 2-AG are involved in up-regulation of the sensitization process remains unclear. The 2-AG may induce or enhance the migration of Langerhans cells, inasmuch as Maestroni (29) demonstrated that 2-AG acts as a chemoattractant for dendritic cells. Alternatively, 2-AG may play an important role in the processing of Ags. Studies on the molecular mechanism by which the CB2 receptor and 2-AG stimulate the function of Langerhans cells are currently in progress in our laboratory.

One striking observation in the present study is that SR144528 markedly suppressed ear swelling as well as the infiltration by eosinophils of the tissues of mice with chronic contact dermatitis (Figs. 9–11), which is an animal model for allergic dermatitis in humans. These observations point to close involvement of the CB2 receptor and 2-AG in the pathogenesis of chronic contact dermatitis.

A remarkable feature of chronic contact dermatitis is that it is accompanied by the infiltration of tissues by eosinophils. A number of endogenous substances have been shown to act as chemotactic agents for eosinophils: RANTES, eotaxin, MCP-2,3,4, and C5a, and several lipid mediators such as platelet-activating factor and 5-oxo-eicosatetraenoic acid. The 2-AG itself also induces the chemotaxis of eosinophils (26). Notably, the levels of RANTES and eotaxin were markedly elevated in the ear with chronic contact dermatitis (153- and 7.7-fold, respectively) (Fig. 10). In contrast, only modest increases were observed in the ear, with contact dermatitis induced by a single challenge with oxazolone (21- and 1.3-fold for RANTES and eotaxin, respectively) (S. Oka and T. Sugiura, unpublished results). Importantly, the application of SR144528 after the sensitization and challenge with oxazolone markedly reduced the levels of RANTES and eotaxin in the ear with chronic contact dermatitis (Fig. 10). Presumably, 2-AG accelerated the production of chemotactic agents for eosinophils such as RANTES and eotaxin, directly or indirectly, thereby amplifying allergic reactions in chronic contact dermatitis, in addition to acting *per se* as a chemoattractant for eosinophils in the milieu of allergic sites. In any case, these results led us to postulate that the CB2 receptor and 2-AG are closely involved and play important roles in various types of allergic reactions in which eosinophils are crucially implicated.

Possible stimulative roles of the CB2 receptor in inflammation and immunity have recently been suggested by several investigators as well. Iwamura et al. (49) reported that several CB2 receptor antagonists or inverse agonists such as JTE-907 and SR144528 reduced carrageenan-induced mouse paw edema, and Ueda et al. (50) demonstrated that the CB2 receptor is closely involved in cutaneous inflammation induced by dinitrofluorobenzene. We also

found that SR144528 markedly suppressed the ear swelling induced by dinitrofluorobenzene in mice (S. Oka and T. Sugiura, unpublished results). In contrast, Jan et al. (51) and Lunn et al. (52) demonstrated that Δ^9 -tetrahydrocannabinol and several CB2 receptor antagonists suppressed inflammatory reactions in the airway. These experimental results are generally consistent with the experimental results obtained in the present study and previously (30), although no information is available on 2-AG in these studies by other investigators (50–52). In any case, it is becoming evident that the CB2 receptor and 2-AG play important roles in a variety of acute and chronic inflammations. Further detailed studies on the CB2 receptor and 2-AG are thus essential for a better understanding of the precise regulatory mechanisms of various inflammatory reactions and immune responses.

In conclusion, we found that the amount of 2-AG was increased in the ear of sensitized mice following a challenge with oxazolone. The CB2 receptor was involved in both the sensitization phase and the elicitation phase of oxazolone-induced contact dermatitis. We also found that the CB2 receptor and 2-AG are closely involved in chronic dermatitis, which is accompanied by the infiltration of tissues by eosinophils. These results strongly suggest that the CB2 receptor and 2-AG play important stimulative roles in the sensitization, elicitation, and exacerbation of allergic inflammation. A thorough elucidation of the pathophysiological significance of the CB2 receptor and 2-AG in inflammatory reactions and immune responses would thus open up new therapeutic approaches to allergic diseases in humans.

Disclosures

The authors have no financial conflict of interest.

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