

Topical Azithromycin and Clarithromycin Inhibit Acute and Chronic Skin Inflammation in Sensitized Mice, with Apparent Selectivity for Th2-Mediated Processes in Delayed-Type Hypersensitivity

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Abstract—Macrolide antibiotics inhibit the secretion of Th1 cytokines while their effects on the release of Th2 cytokines are variable. We investigated molecular and cellular markers of Th1- and Th2-mediated inflammatory mechanisms and the anti-inflammatory activity of azithromycin and clarithromycin in phorbol 12-myristate 13-acetate (PMA) and oxazolone (OXA)-induced skin inflammation. Dexamethasone (50 µg/ear), azithromycin, and clarithromycin (500 µg/ear) reduced TNF-α and interleukin (IL)-1β concentration in ear tissue by inhibiting inflammatory cell accumulation in PMA-induced inflammation. In OXA-induced early delayed-type hypersensitivity (DTH), the macrolides (2 mg/ear) and dexamethasone (25 µg/ear) reduced ear tissue inflammatory cell infiltration and secretion of IL-4 while clarithromycin also decreased IFN-γ concentration. Macrolides showed better activity when administered after the challenge. In OXA-induced chronic DTH, azithromycin (1 mg/ear) reduced the number of ear tissue mast cells and decreased the concentration of IL-4 in ear tissue and of immunoglobulin (Ig)E in serum. Clarithromycin (1 mg/ear) reduced serum IgE concentration, possibly by a mechanism independent of IL-4, while both macrolides attenuated mast cell degranulation. In conclusion, azithromycin and clarithromycin attenuate pro-inflammatory cytokine production and leukocyte infiltration during innate immune reactions, while selectively affecting Th2 rather than Th1 immunity in DTH reactions.

KEY WORDS: macrolide anti-inflammatory activity; oxazolone; PMA; skin inflammation; Th2 cytokines.

INTRODUCTION

Macrolide antibiotics, isolated from Streptomyces, have been used for decades for the treatment of moderate to severe bacterial infections [1]. Some of them also exert anti-inflammatory and immunomodulatory activity [2–4].

Azithromycin and clarithromycin accumulate in inflammatory cells, especially neutrophils and macrophages [5–7], inhibiting the synthesis of reactive oxygen

species and/or secretion of cytokines and chemokines [3, 8–11]. Inhibition of adhesion molecule expression on epithelial cells, with suppression of leukocyte adhesion and infiltration into tissues has also been reported [3, 8, 12]. Although the mechanism of their anti-inflammatory activity remains unknown, certain macrolides inhibit Th1-mediated inflammation, reducing the secretion of pro-inflammatory cytokines such as interleukin (IL)-1, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and granulocyte monocyte colony-stimulating factor (GM-CSF). On the other hand, their effects on Th2 cytokines, such as IL-4 and IL-10 are variable—stimulatory and inhibitory depending on the model, indicating immunomodulation by the macrolides [3, 13].

Up till now, the anti-inflammatory activity of macrolide antibiotics *in vivo* has been investigated

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mainly in animal models of airway inflammation [9, 12, 14–17]. We have also published results on anti-inflammatory effects of azithromycin and clarithromycin in lipopolysaccharide (LPS)-induced endotoxemia and septic shock [18, 19]. Macrolide anti-inflammatory activity in models of skin inflammation *in vivo*, though, has been poorly explored. Oral and topical macrolides have been shown to inhibit ear and paw edema induced by croton oil, cantharidin, oxazolone (OXA), picryl-chloride, and carrageenin [20–22]. However, the molecular and cellular mechanisms of inflammation in these animal models remained unexplored. Hashimoto *et al.* [23] studied the possible mechanism of action of oral clarithromycin in *Staphylococcus aureus*-induced dermatitis in NC mice, but did not distinguish antibacterial from anti-inflammatory effects.

In view of the paucity of information on the mode of action of macrolides in murine experimental skin inflammation, we investigated the mechanisms of modulation by topical standard macrolide antibiotics, azithromycin, and clarithromycin, of (a) innate immune reactions in phorbol 12-myristate 13-acetate (PMA)-induced acute skin inflammation and (b) markers of Th1 and Th2 inflammatory reactions in OXA-induced acute and chronic delayed-type hypersensitivity (DTH).

MATERIALS, METHODS, AND ANIMALS

Animals

PMA-induced acute skin inflammation was performed on CD1 male mice, as described previously [24], while OXA-induced acute and chronic DTH were conducted on male Balb/c mice [25]. All animals were 8–10 weeks old and obtained from Charles River, France. Eight mice per group were kept on wire mesh floors with irradiated maize granulate bedding (Scobis Due, Mucedola, Italy) and maintained under standard laboratory conditions (temperature, 23–24°C; relative humidity, 60±5%; approximately 15 air changes per hour; artificial lighting with circadian cycle of 12 h). Food (Standard food for mice and rats, 4R21, Mucedola, Italy) and tap water were provided *ad libitum*. Mice were allowed to acclimatize for 10 days before the beginning of each experiment. All procedures on animals were performed in accordance with (a) the EEC Council Directive 86/609 of 24th November 1986 on the approximation of laws, regulations, and administrative provisions of the Member States regarding the protection

of animals used for experimental and other scientific purposes; and (b) Statute of Republic Croatia, Animal Welfare Law, NN 135 of 13th December 2006.

Drugs, Chemicals, and Antibodies

Azithromycin was purchased from PLIVA Inc., Croatia and clarithromycin and dexamethasone from Sigma Chemical Co., USA. PMA was obtained from Alexis Biochemicals, USA. OXA was purchased from Sigma Chemical Co., USA. Inhalation anesthetic isoflurane, used in all experiments, was obtained from Forane, Abbott Laboratories Ltd., UK, while thiopental, used for euthanasia of experimental animals, was from Nycomed, Germany. Organic diluents (acetone, isopropanol, and benzyl alcohol) were obtained from Kemika, Croatia. Diff-Quick staining set was purchased from Dade Behring Inc., USA. TNF- α and IL-1 β for enzyme-linked immunosorbent assay (ELISA) were from R&D Systems, USA; IL-4 and IFN- γ from Amersham Biosciences, UK; myeloperoxidase (MPO) from Hycult Biotechnology b. v., Netherlands; and immunoglobulin (Ig)E from Pharmingen, USA. Cell and tissue staining kit for immunohistochemistry was obtained from R&D Systems, USA and antibodies for TNF- α (sc-1351) and IL-1 β (sc-7884) were from SantaCruz Biotechnology. All other reagents were from Sigma Chemical Co., USA.

Experimental Procedures

Anesthesia and Drug Treatment

In all experiments, to apply the irritant and various compounds to the ears, animals were first anesthetized by inhalation of isoflurane, delivered in an anesthesia induction chamber (Stoelting Co., USA). Gas scavenging was provided using the Fluovac 240 V system (International Market Supply, England). Azithromycin, clarithromycin, and dexamethasone, at different concentrations, were applied topically in 15 μ l vehicle (acetone/isopropanol/benzyl alcohol=40:50:10% for PMA experiments, acetone for OXA experiments). Drug application was to the inner surface of the left ear when ear edema was evaluated, or to both ears in chronic OXA-DTH or when ELISA and histopathological analyses in acute models were to be performed. Before taking ear tissue biopsies, animals were killed by intraperitoneal (i.p.) injection of thiopental.

PMA-Induced Acute Skin Inflammation

PMA induction of acute skin inflammation was conducted according to Napoletano *et al.* [24] with some modifications.

In the first experiment, the effect of azithromycin and clarithromycin was assessed on the appearance of ear edema. Animals were topically treated with azithromycin (5–500 µg/ear), clarithromycin (5–500 µg/ear), dexamethasone (50 µg/ear) or vehicle 30 min prior to PMA (0.01%, 12 µl in acetone to the inner surface of the left ear). Six hours after PMA, animals were euthanized and an 8 mm diameter circle was cut with a disposable biopsy punch (Stiefel Laboratories Ltd., Ireland) from the left and right ear of each animal and weighed on a precise analytical balance. Ear edema was assessed as the difference between left (treated) and right (untreated) ear weight.

To determine the time course of TNF-α, IL-1β, and MPO production in response to PMA applied to the inner surface of both ears, animals were euthanized 30 min and 1, 2, 4, 6, 8, 12, 24, and 48 h later. Negative controls received the same volume of acetone. Ear tissue was sampled by cutting both ears at their base and transferring them to Eppendorf tubes to be frozen at –20°C for ELISA.

In the third experiment, animals were treated with azithromycin (500 µg/ear), clarithromycin (500 µg/ear), dexamethasone (50 µg/ear) or vehicle (negative and positive control), 30 min prior to PMA. TNF-α, IL-1β, and MPO concentrations in ear tissue were determined by ELISA, at relevant peak concentration time-points, namely 2 h for TNF-α and 8 h after PMA for IL-1β and MPO. For histological evaluation and immunohistochemical analysis of TNF-α and IL-1β secretion, ears were cut at their base and fixed in 10% buffered neutral formalin fixative 6 and 24 h after PMA.

OXA-Induced Acute Delayed-Type Hypersensitivity

OXA induction of acute DTH was conducted according to Webb *et al.* [25] with some modifications.

For OXA sensitization, the abdominal region was surgically close shaved (Contura cordless clipper, International Market Supply, England), under isoflurane anesthesia, excess loose hair was brushed off and 100 µl of 2% OXA solution in acetone was topically applied to the abdominal surface. Seven days later, animals were challenged by topical administration of 10 µl of 2% OXA solution in acetone to the inner

surface of the ear. Negative controls were sensitized and challenged with acetone.

In the first experiment, the effects were assessed of (a) azithromycin (500 µg, 1 mg and 2 mg/ear), clarithromycin (500 µg, 1 mg and 2 mg/ear), dexamethasone (25 and 50 µg/ear) or vehicle (acetone), given 30 min before OXA challenge or (b) azithromycin (2 mg/ear), clarithromycin (2 mg/ear) or dexamethasone (25 and 50 µg/ear), given 30 min after OXA challenge. Twenty-four hours after challenge, animals were euthanized, ears weighed, and ear edema assessed as described above. The time course of IFN-γ and IL-4 production and histology of cell accumulation in ear dermis, was assessed, as described above, in animals euthanized 0.5, 1, 2, 4, 6, 8, and 24 h after OXA challenge.

In the last experiment, animals were treated with (a) azithromycin (1 mg/ear), clarithromycin (1 mg/ear), dexamethasone (25 µg/ear) or vehicle (negative and positive control) or (b) azithromycin (2 mg/ear) or clarithromycin (2 mg/ear) 30 min after challenge. IFN-γ and IL-4 concentration by ELISA and cell accumulation in ear dermis by histology were assessed 4 h (IFN-γ) and 24 h (IL-4) after inflammation induction.

OXA-Induced Chronic Delayed-Type Hypersensitivity

OXA induction of chronic DTH was conducted according to Webb *et al.* [25] with some modifications.

Seven, nine, 11, 14, 16, 18, 21, 23, 25, 28, 30, and 32 days after abdominal sensitization, anesthetized animals were challenged by topical application of 10 µl of 0.8% OXA solution in acetone to the inner surface of both ears. Negative control received 10 µl acetone. Thirty minutes prior to each challenge, animals were treated with azithromycin (1 mg/ear), clarithromycin (1 mg/ear), dexamethasone (10 µg/ear) or vehicle (acetone) (negative and positive control). Ear thickness was measured 24 h after the 1st, 2nd, 5th, 8th, and 12th challenge, using a digital caliper (Mitutoyo IP63; type 209-530, Japan) connected to a measuring system (Plexx, Netherlands) consisting of the computer program “Windows Hyper Terminal” and a pedal. Immediately after sensitization and 17, 24, and 33 days later (24 h after the 5th, 8th, and 12th challenge), animals were weighed, anesthetized with i.p. thiopental injection and exsanguinated by puncturing the *V. jugularis* and *A. carotis communis*. Blood was collected into tubes with serum separator and centrifuged (room temperature, 2,000×g, 15 min). Serum was transferred into new

tubes and frozen at -20°C for IgE concentration analysis by ELISA. Immediately afterwards, both ears were cut at their base and stored, as described above, for IFN- γ and IL-4 ELISA and histopathological assessment.

ELISA Analysis

TNF- α , IL-1 β , MPO, IFN- γ , and IL-4 concentrations in ear tissue were determined by sandwich ELISA, using capture and detection antibodies according to the manufacturer's recommendations. Both ears from each animal were homogenized in 1 ml of phosphate buffered saline containing protease inhibitors (pepstatin A, phenylmethanesulfonyl fluoride, leupeptin and aprotinin). Homogenate was centrifuged ($10,000\times g$) for 10 min at 4°C . The supernatant was diluted 4-fold for IFN- γ and IL-4, 10-fold for TNF- α and IL-1 β and 100-fold for analysis of MPO concentration. Concentrations were expressed as picogrammes per milligrams of total protein per ear tissue, previously determined by biochemical analyzer (Olympus AU 400, UK).

IgE concentration in serum, diluted 100-fold beforehand, was determined by sandwich ELISA, using capture and detection antibodies according to the manufacturer's recommendations. Concentrations were expressed as nanogrammes per milliliter.

Histopathological Analysis

Ear tissue was fixed in 10% buffered neutral formalin fixative, embedded *in toto*, cut into 3 μm sections and stained routinely with hematoxylin/eosin and Giemsa for mast cells. Inflammatory markers were investigated observer-blind in four sections from each ear specimen.

For immunohistochemistry, goat polyclonal TNF- α antibody (dilution, 1:25) and rabbit polyclonal IL-1 β antibody (dilution, 1:25) and appropriate HRP-DAB Kit were used.

In PMA-induced acute skin inflammation, as well as in OXA-induced acute and chronic DTH, accumulation of mixed populations of polymorphonuclear (PMNL) and mononuclear cells in dermis was assessed and graded according to the following criteria:

- 0 No PMNL/mononuclear cells
- 1 Few scattered PMNL/mononuclear cells
- 2 Larger aggregates of PMNL/mononuclear cells
- 3 Marked accumulation of PMNL/mononuclear cells

In borderline cases, an intermediate grade was used (0–1, 1–2, and 2–3).

In chronic DTH, the total number and percent degranulation of mast cells were also determined.

Data Analysis and Statistical Evaluation

Differences in observed values in all investigated inflammatory markers, between positive control and negative control or groups treated with azithromycin, clarithromycin, or dexamethasone, were analyzed statistically using the Mann–Whitney *U* test. All statistical analyses were done using the program GraphPad InStat v. 4.0 (GraphPad Software Inc., USA). The level of significance was set at $p < 0.05$ in all cases.

RESULTS

PMA-Induced Acute Skin Inflammation

Topical application of azithromycin (100, 250, and 500 $\mu\text{g}/\text{ear}$), clarithromycin (50, 100, 250, and 500 $\mu\text{g}/\text{ear}$) as well as dexamethasone at 50 $\mu\text{g}/\text{ear}$ significantly reduced ear edema at 6 h after PMA (Fig. 1). The two macrolides achieved around 50% inhibition at 500 $\mu\text{g}/\text{ear}$.

Concentrations of IL-1 β and TNF- α were significantly raised for 2 to 12 h after PMA. While TNF- α failed to increase beyond 2 h ($1,012.2 \pm 32$ pg/mg protein), IL-1 β increased markedly to a peak at 8 h ($12,231.1 \pm 1,561.3$ pg/mg protein). Concentration of MPO reached a peak at 8 h ($17,005.3 \pm 3,452.1$ pg/mg protein) and remained high for 24–48 h. Both azithromycin and clarithromycin at doses of 500 $\mu\text{g}/\text{ear}$, as well as dexamethasone at 50 $\mu\text{g}/\text{ear}$, significantly reduced peak cytokine and MPO concentrations in ear tissue, the most pronounced effects being observed on MPO (Table 1).

Histopathological and immunohistochemical analysis of ear tissue samples revealed the presence, in positive controls, of edema with marked accumulation of predominantly PMNL and a few mononuclear cells in the dermis 6 h after PMA application (Fig. 2a, b). IL-1 β and TNF- α were secreted mainly by accumulated PMNL (data not shown). Twenty-four hours after PMA, edema was reduced and although the accumulation of PMNL and mononuclear cells was pronounced, the majority of these cells were negative for IL-1 β and TNF- α secretion (data not shown). Azithromycin and clarithromycin (500 $\mu\text{g}/\text{ear}$) and dexamethasone (50 $\mu\text{g}/\text{ear}$) suppressed edema appearance and reduced IL-1 β and

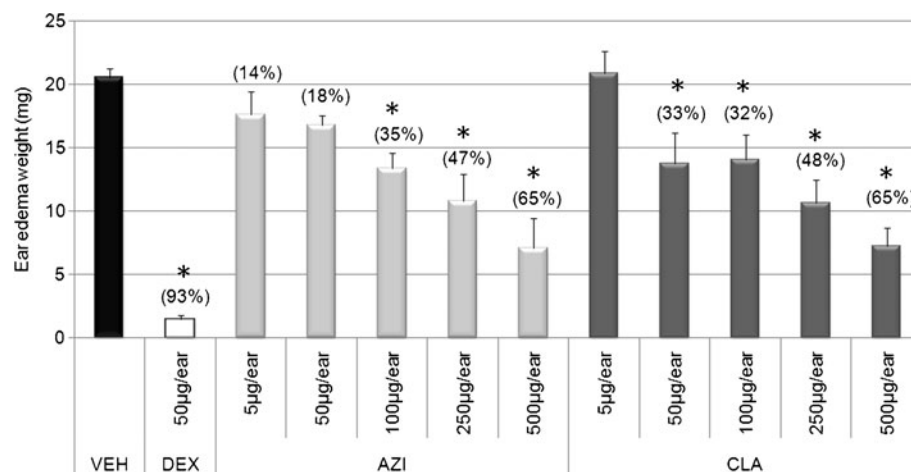


Fig. 1. Ear edema weight 6 h after PMA administration. Dexamethasone (DEX), azithromycin (AZI), clarithromycin (CLA), and vehicle (VEH) were applied topically 30 min before inflammation induction. Ear edema is presented as difference in weight between treated and non-treated ear. Numbers in brackets represent percent inhibition in comparison to the vehicle-treated group. Data are presented as group means $\pm$ SEM. * $p < 0.05$ versus vehicle-treated group; $n = 8$ per group.

TNF- α secretion in ear tissue 6 h after PMA application predominantly by decreasing the accumulation of dermal PMNL (Figs. 2c–e and 3). By 24 h after inflammation induction, responses to all the administered substances were no longer observable (Fig. 3).

OXA-Induced Acute Delayed-Type Hypersensitivity

In mice sensitized with OXA, azithromycin and clarithromycin (0.5, 1, and 2 mg/ear) and dexamethasone (25 and 50 μ g/ear), administered either before or after challenge with OXA, all significantly reduced ear edema at 24 h post-challenge (Fig. 4). Macrolides demonstrated better activity when administered after the challenge, although this difference was not statistically significant. Azithromycin, at all times, was somewhat more effective than clarithromycin.

Sensitization and challenge with OXA significantly increased concentrations of the Th1 cytokine, IFN- γ , and the Th2 cytokine, IL-4, in ear tissue. IFN- γ was significantly increased for 0.5–8 h, with a peak at 4 h after challenge (445.4 ± 24.8 pg/mg protein) and IL-4 was significantly elevated for 2–24 h, reaching a peak at 24 h after OXA challenge (131.5 ± 12.4 pg/mg protein).

Like dexamethasone (25 μ g/ear), neither azithromycin nor clarithromycin (1 mg/ear), given 30 min before challenge, altered ear 4 h peak IFN- γ concentrations; only clarithromycin (2 mg/ear) inhibited IFN- γ when applied 30 min after challenge (Fig. 5). In contrast, while dexamethasone (25 μ g/ear), and not the two macrolides, inhibited ear 24 h peak IL-4 concentrations, when applied before OXA challenge, both azithromycin and clarithromycin effectively inhibited ear IL-4, when applied 30 min after OXA challenge.

Table 1. Effects of dexamethasone, azithromycin, and clarithromycin on interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), and myeloperoxidase (MPO) concentration in mouse ear tissue 8 h (IL-1 β and MPO) and 2 h (TNF- α) after PMA administration

Substance administered	Concentration (pg/mg protein;% inhibition) ^a		
	IL-1 β	TNF- α	MPO
Negative control	182 $\pm$ 22*	662 $\pm$ 43*	72 $\pm$ 10*
Positive control	1,080 $\pm$ 2,320	958 $\pm$ 120	16,872 $\pm$ 4,628
Dexamethasone (50 μ g/ear)	3,871 $\pm$ 511 (64)*	576 $\pm$ 58 (40)*	4,798 $\pm$ 1,519 (72)*
Azithromycin (500 μ g/ear)	6,181 $\pm$ 741 (42)*	480 $\pm$ 62 (49)*	4,324 $\pm$ 921 (74)*
Clarithromycin (500 μ g/ear)	4,390 $\pm$ 481 (59)*	475 $\pm$ 59 (50)*	4,101 $\pm$ 898 (76)*

^a Data are presented as group means $\pm$ SEM; $n = 8$ per group

* $p < 0.05$ versus appropriate positive control

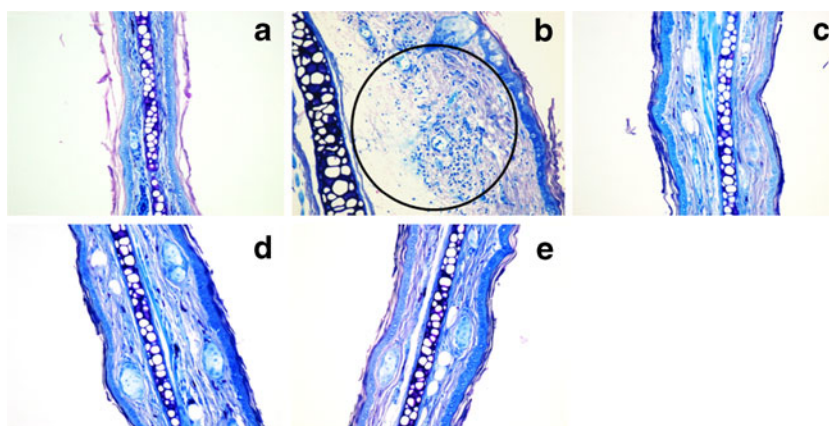


Fig. 2. Representative photomicrographs of mouse ear tissue (hematoxylin/eosin and Giemsa, $\times 200$). **a** Ear tissue from a healthy negative control animal. Six hours after topical PMA application, accumulation of polymorphonuclear (PMNL) and mononuclear cells, as well as edema were observed (**b**). The circle indicates the edematous dermis in the positive control ear filled with PMNL and mononuclear cells. Dexamethasone (**c**), azithromycin (**d**), and clarithromycin (**e**), all decreased accumulation of PMNL and mononuclear cells, as well as edema 6 h after PMA application.

Histopathological analysis of ear tissue showed that, in comparison to the healthy ear (Fig. 6a), a mixed population of PMNL and mononuclear cells started to infiltrate the ear dermis 4 h after OXA challenge.

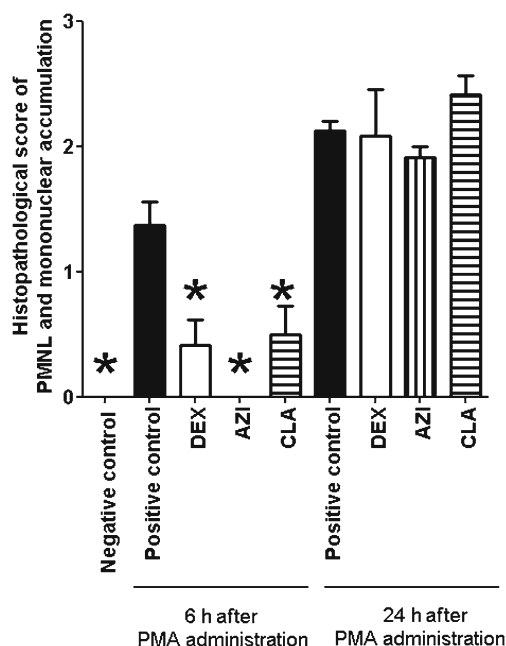


Fig. 3. Azithromycin (AZI; 500 $\mu\text{g}/\text{ear}$), clarithromycin (CLA; 500 $\mu\text{g}/\text{ear}$) and dexamethasone (DEX; 50 $\mu\text{g}/\text{ear}$), administered 30 min before topical PMA application, significantly decreased polymorphonuclear (PMNL) and mononuclear cell accumulation 6 h after inflammation induction. Substances had no effect on the accumulation of inflammatory cells 24 h after inflammation induction. Data are presented as group median $\pm$ range of histological scores of mixed PMNL and monocyte infiltration into ear dermis. * $p < 0.05$ versus vehicle-treated positive control; $n = 8$ per group.

Marked, increasing accumulation of these cells in ear dermis was observed at 8 h and reached its peak 24 h after OXA challenge (Fig. 6b). Azithromycin and clarithromycin, applied after challenge at 2 mg/ear, as well as dexamethasone (25 $\mu\text{g}/\text{ear}$) administered before challenge, significantly reduced accumulation of PMNL and mononuclears 24 h after OXA challenge (Figs. 6c–e and 7). Administered 30 min before the OXA challenge, the two macrolides (1 mg/ear) had no effect on the accumulation of inflammatory cells (Fig. 7).

OXA-Induced Chronic Delayed-Type Hypersensitivity

Sensitization and multiple challenges of mice with OXA provoked a biphasic, sustained ear edema, with a small peak at 8 days and a continued increase in edema from 10–33 days (Fig. 8). Azithromycin and clarithromycin at 1 mg/ear and dexamethasone at 10 $\mu\text{g}/\text{ear}$, each administered topically 30 min before each challenge, markedly reduced ear edema in the order, dexamethasone $\geq$ azithromycin $>$ clarithromycin (Fig. 8).

Multiple challenges with OXA significantly increased concentrations of the Th1 cytokine, IFN- γ , 17–24 days after sensitization, as well as of the Th2 cytokine, IL-4, which were prolonged for 17–33 days after sensitization. Concentrations of IFN- γ reached a peak on day 24 (247.5 ± 19.2 pg/mg protein) and of IL-4 on day 17 (58 ± 8.2 pg/mg protein). Serum IgE concentrations progressively increased until the end of the experiment, 33 days after OXA sensitization ($20,283.8 \pm 2,026.8$ ng/ml). Investigation of the effects of azithromycin

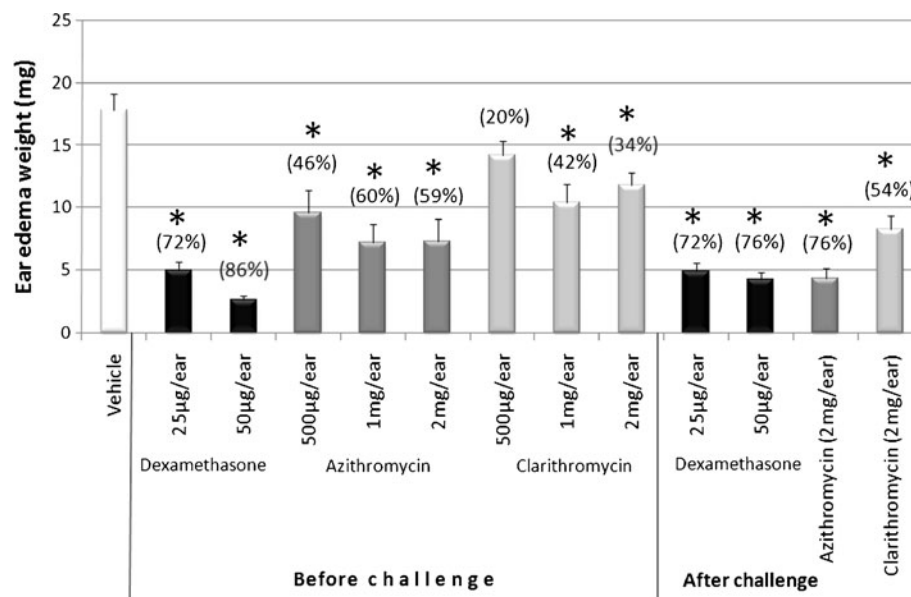


Fig. 4. Effects of dexamethasone, azithromycin, and clarithromycin, applied 30 min before or 30 min after oxazolone (OXA) challenge in OXA-induced acute delayed-type hypersensitivity model, on ear edema weight, measured 24 h post-challenge. Ear edema is presented as the difference in weight between treated and non-treated ears. Numbers in brackets represent percent inhibition in comparison to the vehicle-treated group. Data are presented as group means $\pm$ SEM. * $p < 0.05$ versus vehicle-treated group; $n = 8$ per group.

and clarithromycin on cytokine and IgE production was limited to time-points at which the peak concentrations of these mediators were measured. In contrast to dexamethasone (10 μ g/ear), which significantly increased the concentration of IFN- γ (by 49%), azithromycin, and clarithromycin (1 mg/ear) had no marked effect on the concentration of the Th1 cytokine, IFN- γ , measured on day 24 after OXA sensitization (Table 2). Significant inhibition of production of the Th2 cytokine, IL-4, was

achieved only by azithromycin, not dexamethasone or clarithromycin, on day 17 after OXA sensitization (Table 2). Unlike dexamethasone, both topically administered macrolides markedly decreased IgE concentrations in serum, measured 33 days after OXA sensitization (Table 2).

Histopathological analysis revealed progressive accumulation of PMNL, mononuclear cells and mast cells in the ear dermis of vehicle-treated animals until

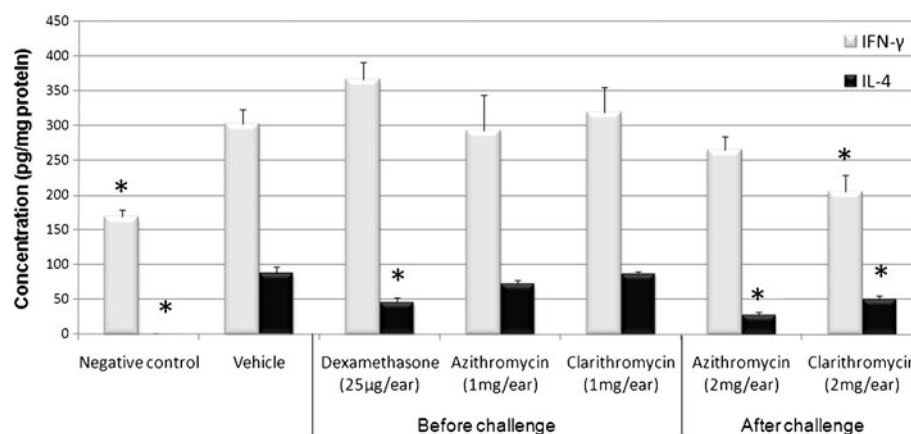


Fig. 5. Effects of dexamethasone, azithromycin, and clarithromycin, applied 30 min before or 30 min after oxazolone (OXA) challenge, on interferon- γ (IFN- γ) and interleukin-4 (IL-4) concentrations in mouse ear tissue 4 h (IFN- γ) and 24 h (IL-4) after OXA administration in OXA-induced acute delayed-type hypersensitivity mode. Data are presented as group means $\pm$ SEM. * $p < 0.05$ versus appropriate vehicle-treated group; $n = 8$ per group.

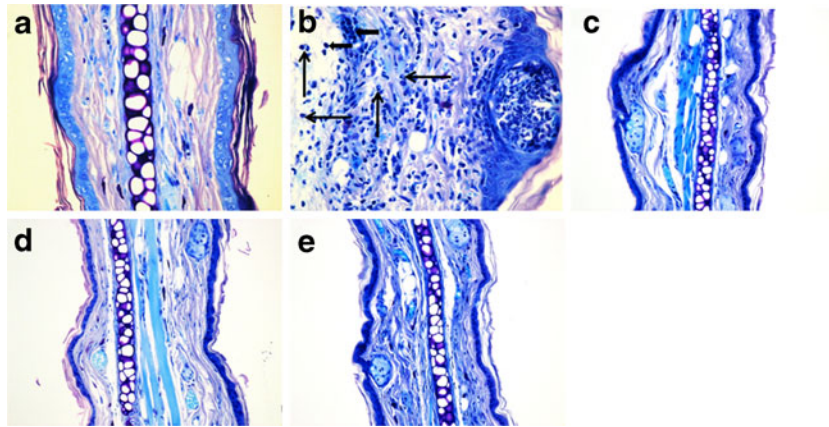


Fig. 6. Representative photomicrographs of mouse ear tissue (hematoxylin/eosin and Giemsa, $\times 400$) after topical oxazolone (OXA) challenge in OXA-induced acute delayed-type hypersensitivity model. Thin arrows indicate polymorphonuclear cells (PMNL); thick arrows indicate mononuclear cells. **a** Ear tissue from a healthy animal in the negative control group. Mononuclear and PMNL cell accumulation progressively increased 24 h after challenge (**b**). Dexamethasone (**c**), azithromycin (**d**), and clarithromycin (**e**) decreased PMNL and mononuclear cell accumulation 24 h after challenge.

the end of the experiment, 33 days after OXA sensitization (Fig. 9a, b). Only dexamethasone (10 $\mu\text{g}/\text{ear}$), not the two macrolides, significantly reduced the number of PMNL and mononuclear cells, at 33 days after sensitization (Figs. 9c–e and 10). The total number of mast cells was significantly decreased by azithromycin (1 mg/ear), 17 days after sensitization and by dexamethasone, 24 and

33 days after sensitization (Fig. 11a). However, unlike dexamethasone, the two macrolides (1 mg/ear) significantly inhibited degranulation of mast cells 24 days after OXA sensitization, when the extent of mast cell degranulation in vehicle-treated, inflamed skin had reached its peak (Fig. 11b).

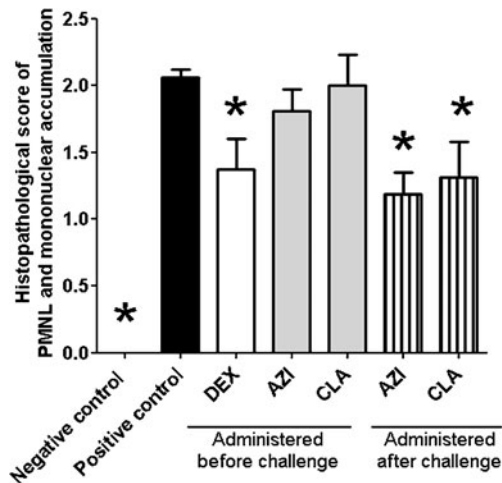


Fig. 7. Azithromycin (AZI) and clarithromycin (CLA), administered 30 min after oxazolone (OXA) challenge at 2 mg/ear, as well as dexamethasone (DEX; 25 $\mu\text{g}/\text{ear}$), significantly decreased polymorphonuclear (PMNL) and mononuclear cell accumulation 24 h after challenge in OXA-induced acute delayed-type hypersensitivity model. When administered before the challenge, azithromycin and clarithromycin, both at 1 mg/ear, had no effect on the accumulation of inflammatory cells. Data are presented as group median $\pm$ range of histological scores of mixed PMNL and monocyte infiltration into ear dermis 24 h after OXA challenge. * $p < 0.05$ versus vehicle-treated positive control, $n = 8$ per group.

DISCUSSION AND CONCLUSIONS

The data presented here confirm that like dexamethasone, the topically applied macrolides, azithromycin, and clarithromycin, inhibit acute inflammatory responses, including leukocyte accumulation and mediator production. While less effective than dexamethasone at inhibiting inflammation in acute OXA-DTH, when given before OXA challenge, the two macrolides, effectively inhibited acute dermal inflammation and IL-4 production when given after challenge. In chronic OXA-DTH, azithromycin was particularly effective at inhibiting skin edema, dermal IL-4, serum IgE, and mast cell degranulation.

The results in acute PMA-induced ear edema confirm previous findings that macrolide antibiotics are effective anti-inflammatory agents in such acute experimental dermal inflammatory models. Tarayre *et al.* [22] reported that a single dose of topically applied erythromycin (0.250–1 mg/ear) markedly decreases ear edema induced in the mouse by croton oil, cantharidin, and picryl-chloride. Agen *et al.* [20] also found that a single oral dose of roxithromycin at 5 and 20 mg/kg significantly reduces croton oil, as well as carrageenin

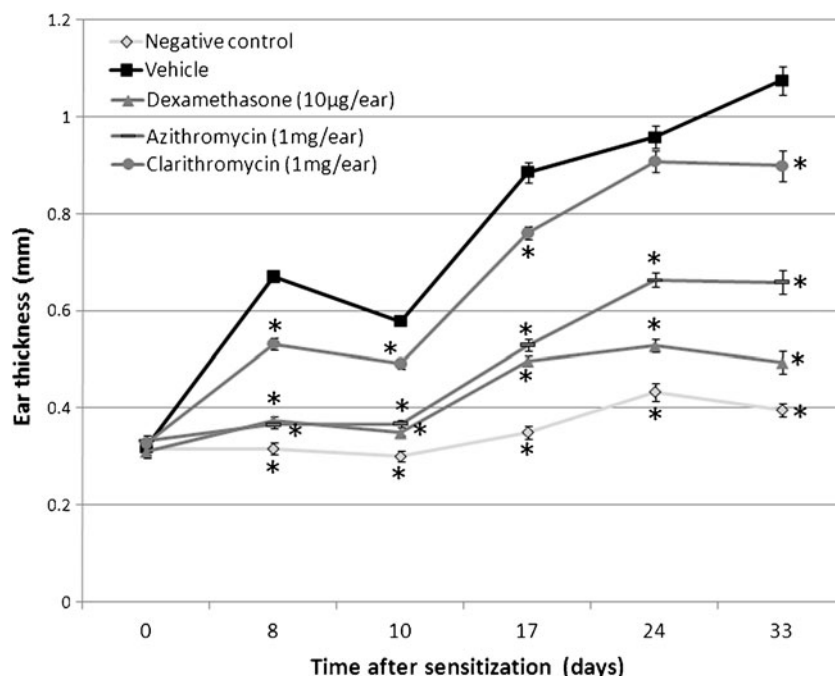


Fig. 8. Effects at different time-points after oxazolone (OXA) sensitization of multiple topical treatments of mice ears with azithromycin, clarithromycin, and dexamethasone, applied 30 min before each OXA challenge, on ear thickness measured 24 h after the challenge in OXA-induced chronic delayed-type hypersensitivity model. Data are presented as group means $\pm$ SEM. * $p < 0.05$ versus vehicle-treated group at same time point; $n = 8$ per group.

and poly-L-arginine induced paw edema. Subsequently, Scaglione and Rossoni [21] indicated that single oral pretreatment with roxithromycin, azithromycin, and clarithromycin at 20 mg/kg markedly inhibits carrageenin induced paw edema in the rat. But none of these authors explored the mechanisms involved.

Epicutaneous application of PMA results in vascular leakage, leukocyte infiltration, epidermal hyperplasia, activation of protein kinase C and increased release of arachidonic acid and its metabolites [26]. The capacity of azithromycin and clarithromycin to exert broad anti-inflammatory effects, similar to those of dexamethasone, was confirmed by the inhibition by all three agents of edema, leukocyte infiltration, as well as IL-1 β and TNF- α and MPO, a marker for PMNLs, in ear tissue of PMA-treated mice (Figs. 1–3; Table 1). Although some leukocyte infiltration was still present after 24 h, edema had started to decline at this time, when immunohistochemical staining of the infiltrated leukocytes for IL-1 β and TNF- α was absent. It seems reasonable to conclude, therefore, that all three tested agents were acting to reduce pro-inflammatory cytokine and other mediator production by infiltrating leukocytes. Such actions have been widely reported for macrolide antibiotics [4, 8, 16, 27].

The ability of macrolide antibiotics to inhibit innate immunity *in vivo*, independently of direct antibacterial actions, is also now well documented. For instance, Bosnar *et al.* [28] recently demonstrated that azithromycin and clarithromycin decrease total cell and neutrophil numbers in bronchoalveolar lavage fluid, lung tissue MPO, IL-1 β , GM-CSF, and TNF- α concentrations in bacterial LPS-induced murine lung neutrophilia. We have also described inhibition by azithromycin and clarithromycin of plasma TNF- α levels in LPS-induced endotoxemia in mice [18, 19].

Little is known, however, about the relative effects of macrolide antibiotics on Th1 and Th2-mediated adaptive immune responses. PMA does not induce formation of IL-4 or IFN- γ in mouse skin [25], but OXA-induced acute and chronic DTH appears to be well suited as a model for this purpose, as markers of both Th1 and Th2 responses can be assessed in the same system. Several authors have shown that topical OXA challenge induces ear edema with infiltration of inflammatory cells by triggering a Th1-mediated inflammatory reaction, observed by increases in IFN- γ and TNF- α concentration. But also Th2-mediated inflammatory reactions can be detected, seen as increases in IL-4 and

Table 2. Effects of multiple topical ear treatments with azithromycin, clarithromycin, and dexamethasone, each applied 30 min before each OXA challenge, on concentrations, measured 24 h after oxazolone (OXA) challenge, of ear tissue interferon- γ (IFN- γ) at 17 days, ear tissue interleukin-4 (IL-4) at 24 days, as well as serum immunoglobulin (Ig)E at 33 days after OXA sensitization in OXA-induced chronic delayed-type hypersensitivity model

Substance administered	Concentration (% inhibition) ^a		
	pg/mg protein		ng/ml IgE
	IFN- γ	IL-4	
Negative control	76 $\pm$ 7*	0*	1,054 $\pm$ 166*
Positive control	247 $\pm$ 19	58 $\pm$ 8	20,284 $\pm$ 2,027
Dexamethasone (10 μ g/ear)	367 $\pm$ 33 ((-48)*)	46 $\pm$ 4 (21)*	21,690 $\pm$ 1,611 ((-7)
Azithromycin (1 mg/ear)	187 $\pm$ 14 (25)	36 $\pm$ 6 (37)*	9,428 $\pm$ 953 (54)*
Clarithromycin (1 mg/ear)	170 $\pm$ 11 (31)	66 $\pm$ 6 ((-14)	13,335 $\pm$ 1,261 (34)*

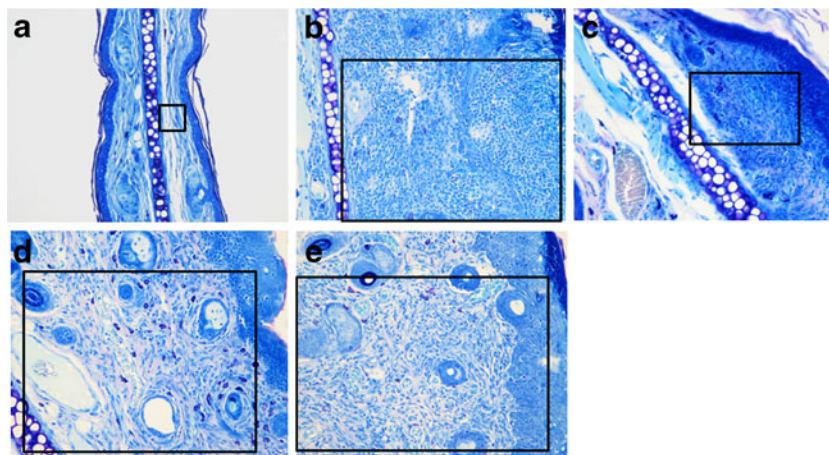
^a Data are presented as group means $\pm$ SEM; $n=8$ per group* $p<0.05$ versus appropriate positive control

IL-10 concentration in ear tissue and serum IgE concentration [25, 29, 30]. Webb *et al.* [25] have shown that the acute response to OXA is predominantly a Th1-mediated process, while with chronic, repeated challenge a Th2-mediated response develops. Using mast cell-deficient mice, they ruled out a crucial role of mast cells as a source of IL-4, and proposed that in the chronic model, infiltrating Th2 lymphocytes are the major producers of IL-4. Nevertheless, in view of the importance of mast cells as a source of IL-4 [31, 32], we included their histological evaluation in our study.

Although, after OXA challenge, we detected the production in ear tissue of both IFN- γ and IL-4, in association with developing inflammation and leukocyte infiltration, there was a clear distinction in the time courses of their release. In the acute model, IFN- γ

release preceded that of IL-4 by 2–4 h, and in the chronic model, IFN- γ was released in larger quantities, but IL-4 release was sustained longer and would have contributed to the gradual increase in ear mast cell accumulation and serum IgE from day 17 [32, 33]. The ear mast cells clearly bound increasing amounts of circulating IgE on their surface, as indicated by the degree of (histologically detected) degranulation in the ear from days 17–24.

In agreement with the response to a single dose of erythromycin (2.5 mg/ear), in acute OXA-induced ear edema [22], azithromycin, and clarithromycin, as well as dexamethasone, significantly decreased acute OXA-induced ear swelling, irrespective of whether administered before or after OXA challenge (Fig. 4). It seems likely that this action was related to direct inhibitory

**Fig. 9.** Representative photomicrographs of mouse ear tissue (hematoxylin/eosin and Giemsa, $\times 200$) in healthy animals in **a** negative control group and **b** vehicle-, **c** dexamethasone-, **d** azithromycin-, and **e** clarithromycin-treated animals 33 days after oxazolone (OXA) sensitization in OXA-induced chronic delayed-type hypersensitivity model. The square indicates ear dermis filled with polymorphonuclear cells, mononuclear cells, and mast cells.

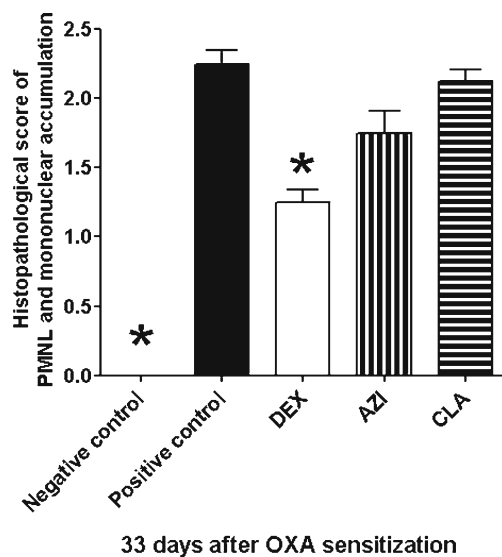


Fig. 10. Effects of multiple treatments with dexamethasone (DEX), azithromycin (AZI), and clarithromycin (CLA), 30 min before each oxazolone (OXA) challenge, on polymorphonuclear (PMNL) and mononuclear cell accumulation in mouse ear tissue 33 days after OXA sensitization in OXA-induced chronic delayed-type hypersensitivity model. Data are presented as group median $\pm$ range of histological scores of mixed PMNL and monocyte infiltration into ear dermis. * $p < 0.05$ versus vehicle-treated positive control; $n = 8$ per group.

effects of the macrolides on endothelial permeability, since, given before challenge, neither azithromycin nor clarithromycin inhibited subsequent OXA challenge-induced leukocyte infiltration nor IFN- γ and IL-4 production (Figs. 5 and 7). However, applied after the OXA challenge, both azithromycin and clarithromycin, like dexamethasone, markedly inhibited leukocyte infiltration into ear tissue (Figs. 6 and 7). The reduction of ear IL-4 concentrations by both macrolides, when applied after OXA challenge, indicates that infiltrating activated Th2 lymphocytes could well have been the targets. Clarithromycin has been reported to exert weak inhibitory effects on IL-4 production by mitogen stimulated human T cells *in vitro* [34] and in our acute DTH model *in vivo* it also reduced the IFN- γ concentration in the ear, indicating non-selective inhibition of infiltrating T cells.

The increasing role of IL-4-producing Th2 lymphocytes with the progression of murine chronic OXA-DTH, as reported by Webb *et al.* [25] and indicated by the prolonged ear IL-4 and serum IgE increases in our study, probably accounts for the fact that, in contrast to dexamethasone, azithromycin inhibited IL-4, but not IFN- γ production in this model. Both macrolides

decreased the raised serum IgE levels (Table 2). Similar effects of roxithromycin (250 $\mu\text{g/kg/day}$ i.p. for 14 days) were seen by Kawazu *et al.* [35] in murine dinitrophenyl-ovalbumin-induced systemic DTH. Recently, clarithromycin (200 mg/kg, i.p.) was also reported to reduce production of IL-4 and IL-5 and inhibit mouse ovalbumin-induced airway inflammation and hyperresponsiveness [36].

A pronounced inhibitory action of azithromycin on Th2 activity from one week after OXA sensitization, would be expected, since the macrolide (1 mg/ear), for several weeks, was as effective as dexamethasone (10 $\mu\text{g/ear}$) at inhibiting ear edema (Fig. 8). However, this effect of azithromycin was not accompanied by clear inhibition of inflammatory cell accumulation in the ear (Figs. 9 and 10), suggesting that in the chronic DTH model, other cells than leukocytes may also have been the targets for the macrolides. While the reduced serum IgE levels do point to a macrolide action on Th2 cells, it seems likely that mast cells, which are also an important source of IL-4 [32], were additional targets. Azithromycin, at 17 days after OXA sensitization, significantly reduced ear tissue mast cell number (Fig. 11a), which likely contributed to its anti-inflammatory effects.

Clarithromycin, however, had no effect on mast cell number or on IL-4 concentration in ear tissue, although it did markedly reduce serum IgE concentration (Table 2; Fig. 11a). In human atopic dermatitis, with which murine chronic OXA-DTH bears some resemblance [25], high IgE titres are not necessarily totally dependent on IL-4 [37]. Therefore, it is possible that clarithromycin reduced serum IgE concentration by IL-4-independent mechanisms. Since macrolides, particularly azithromycin, have been reported to modify monocyte phenotype [38], it is intriguing to speculate whether the apparently preferential effect of these agents on Th2 responses may be related indirectly to macrolide modulation of the interaction between antigen-presenting, activated macrophages or dendritic cells and Th activation.

Binding of specific antigen to IgE antibodies attached to the mast cell surface induces their immediate degranulation, secretion of vasoactive amines, and resulting inflammatory reactions [31]. In contrast to dexamethasone (10 $\mu\text{g/ear}$), topical azithromycin and clarithromycin (1 mg/ear) both markedly reduced peak mast cell degranulation in mouse ear tissue on day 24 after OXA sensitization (Fig. 11b). This clear action undoubtedly contributed to the reduction in ear swelling, to which histamine would have made a major contribution and may help to explain why the macrolides

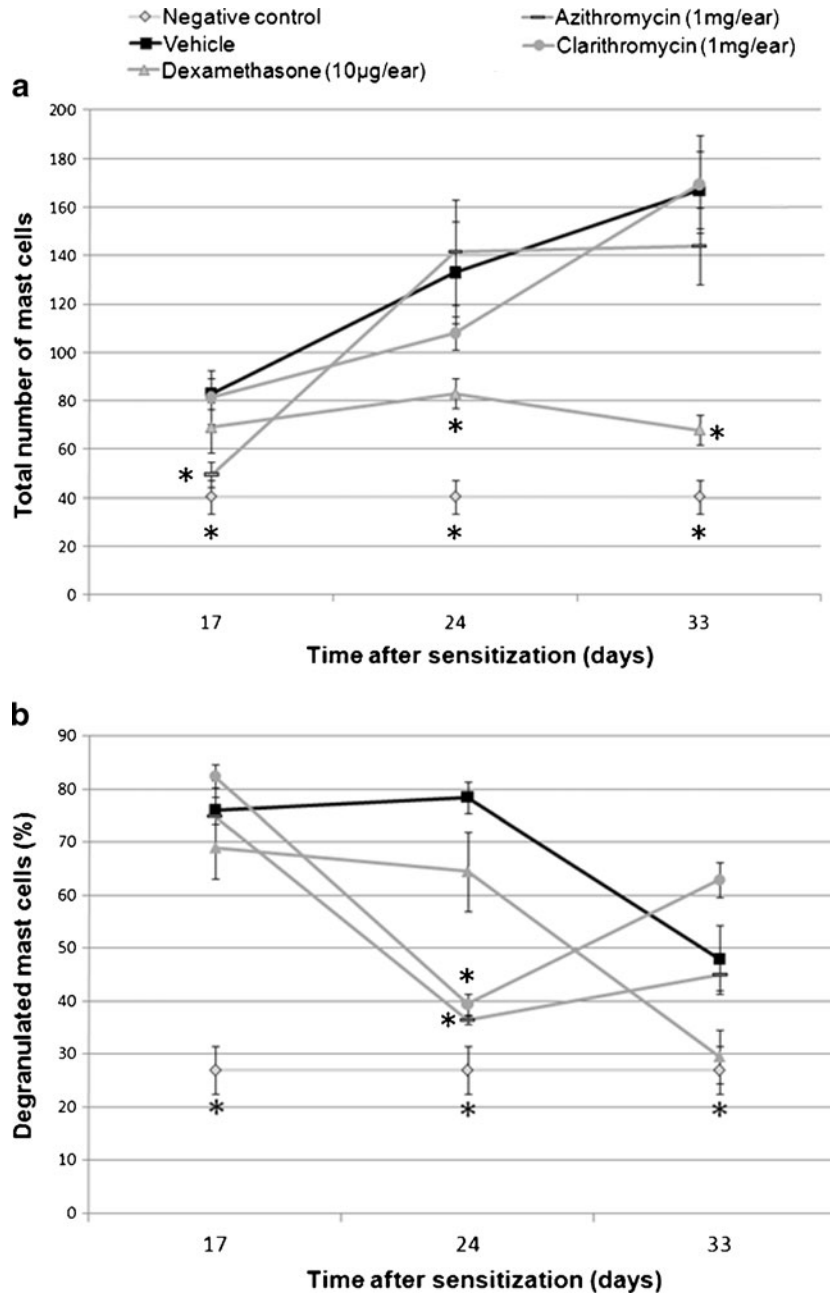


Fig. 11. Effects of multiple treatments with dexamethasone, azithromycin, and clarithromycin (30 min before each oxazolone (OXA) challenge) on total number of mast cells (a) and percentage of degranulated mast cells (b) in mouse ear tissue at indicated time points after OXA sensitization in OXA-induced chronic delayed-type hypersensitivity model. Data are presented as group means $\pm$ SEM. * $p < 0.05$ versus appropriate vehicle-treated group at the same time point; $n = 8$ per group.

inhibited chronic OXA-DTH induced ear swelling, with little effect on leukocyte accumulation. A similar reduction by orally administered clarithromycin (100 mg/kg, three times per week for 9 weeks) of the number of degranulated mast cells in the skin was also observed in NC/Nga mice

infected with *S. aureus* which develop dermatitis, with infiltration of mast cells and MHC class II-positive cells in the skin [23]. It is unclear, though, whether clarithromycin, under these conditions, was acting as an antibacterial or anti-inflammatory agent.

In conclusion, we have defined more precisely the potential targets of action of topical macrolides in murine experimental skin inflammation, particularly that induced by immediate and DTH reactions. In acute inflammation caused by PMA, azithromycin and clarithromycin inhibited pro-inflammatory cytokines and leukocyte infiltration, while in OXA-induced DTH, actions on Th2 lymphocytes, IL-4 production, and mast cell degranulation distinguish the macrolides from corticosteroids such as dexamethasone. These actions of macrolides may provide additional therapeutic benefit in infections involving hypersensitivity to infective pathogens and also suggest potential new directions for inhibitors of skin inflammation.

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