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Contribution of $\alpha_4\beta_1$ integrin to the antiallergic effect of levocabastine

Ahmed R. Qasem [a,1], *Claudio Bucolo* [b,1], *Monica Baiula* [c,1], *Antonino Spartà* [c], *Paolo Govoni* [d], *Andrea Bedini* [c], *Domenico Fascì* [c], *Santi Spampinato* [c,*]

[a] *Department of Medicine, Health Science Campus, University of Toledo, OH, USA*
[b] *Department of Pharmacology, University of Catania, Catania, Italy*
[c] *Department of Pharmacology, University of Bologna, Irnerio 48, 40126 Bologna, Italy*
[d] *Department of Experimental Medicine-Section of Histology, University of Parma, Italy*

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ABSTRACT

Levocabastine is an antiallergic drug acting as a histamine H1-receptor antagonist. In allergic conjunctivitis (AC), it may also antagonize up-regulation of the intercellular adhesion molecule-1 (ICAM-1) expressed on epithelial conjunctival cells. However, little is known about its effects on eosinophils, important effector cells in AC. The adhesion molecule integrin $\alpha_4\beta_1$ is expressed in eosinophils; it interacts with the vascular cell adhesion molecule-1 (VCAM-1) and fibronectin (FN) in vascular endothelial cells and contributes to eosinophil activation and infiltration in AC. This study provides evidence that in a scintillation proximity assay levocabastine (IC_{50} 406 μM), but not the first-generation antihistamine chlorpheniramine, displaced ^{125}I-FN binding to human integrin $\alpha_4\beta_1$ and, in flow cytometry analysis, levocabastine antagonized the binding of a primary antibody to integrin α_4 expressed on the Jurkat cell surface. Levocabastine, but not chlorpheniramine, binds the $\alpha_4\beta_1$ integrin and prevents eosinophil adhesion to VCAM-1, FN or human umbilical vascular endothelial cells (HUVEC) *in vitro*. Similarly, levocabastine affects $\alpha_L\beta_2$/ICAM-1-mediated adhesion of Jurkat cells. In a model of AC levocabastine eye drops reduced the clinical aspects of the late-phase reaction and the conjunctival expression of $\alpha_4\beta_1$ integrin by reducing infiltrated eosinophils. We propose that blockade of integrin-mediated cell adhesion might be a target of the antiallergic action of levocabastine and may play a role in preventing eosinophil adhesion and infiltration in AC.

* *Corresponding author*. Tel.: +39 0512091851; fax: +39 051248862.
E-mail address: santi.spampinato@unibo.it (S. Spampinato).

[1] Contributed equally to this work.

Abbreviations: AC, allergic conjunctivitis; BIO-1211, (*N*-[[4-[[[(2-methylphenyl)amino]carbonyl]amino]-phenyl]acetyl]-L-leucyl-L-aspartyl-L-valyl-L-proline); CMFDA, chloromethylfluorescein diacetate; EPR, early-phase reaction; FACS, flow cytometry analysis; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FN, fibronectin; HBSS, Hank's balanced salt solution; HUVEC, human umbilical vascular endothelial cells; ICAM-1, intercellular adhesion molecule-1; LFA-1, leukocyte function-associated antigen-1; LPR, late-phase reaction; MFI, mean fluorescence intensity; PMSF, phenylmethanesulfonyl fluoride; PSA, ammonium persulfate; PVT, polyvinyltoluene; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SPA, scintillation proximity assay; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; VCAM-1, vascular cell adhesion molecule-1; VKC, vernal keratoconjunctivitis; VLA-4, very late antigen-4.

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1. Introduction

Levocabastine is a potent, selective histamine H_1-receptor antagonist [1,2], employed for the topical treatment of allergic conjunctivitis (AC). Histamine-stimulated phosphatidylinositol turnover and cytokine secretion by human conjunctival epithelial cells are attenuated by levocabastine [3]; however, antihistamine potency alone does not predict its full anti-inflammatory potential. It has in fact been reported that topical levocabastine reduces the expression of the intercellular adhesion molecule-1 (ICAM-1), on epithelial conjunctival cells, *in vivo* and *in vitro* [4,5]. Other anti-allergic agents too, such as fexofenadine [6], cetirizine and olopatadine [7] or azelastine [8] may also reduce ICAM-1 expression.

Increased levels of cell adhesion molecules on the microvasculature and factors that regulate them are likely to be responsible for the conjunctival infiltration of circulating cells and may perpetuate inflammation in AC [9,10]. The central mechanism in the pathogenesis of this disease is IgE-mediated mast cell degranulation and infiltration and activation of T lymphocytes, basophils, eosinophils, and other cells in the conjunctiva, with the involvement of conjunctival epithelial cells [11]. Its severity correlates with the degree of eosinophil infiltration into the conjunctiva, which is mediated by chemokines that stimulate the production of adhesion molecules like ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) on the endothelial cell surface [9,10].

The mechanisms regulating eosinophil adhesion to the ocular surface during allergic inflammation are still largely unknown. The $\alpha_4\beta_1$ integrin, also known as very late antigen-4 (VLA-4), is expressed in eosinophils and contributes to the eosinophil activation and infiltration in AC [9]. $\alpha_4\beta_1$ Integrin-expressing cells infiltrate the conjunctiva and the interaction between $\alpha_4\beta_1$ integrin and VCAM-1 or fibronectin (FN), which are expressed on vascular endothelial cells [12], contributes to both eosinophil rolling and firm adhesion [13–16].

Integrins are composed of α and β subunits and function as both adhesion and signaling molecules [10]. The β_1 subunit's functional status determines the number of eosinophils firmly adherent to the endothelium [9]. Moreover, integrins containing the β_2 subunit, e.g. the $\alpha_L\beta_2$ integrin [leukocyte function-associated antigen-1 (LFA-1)] can bind to ICAM-1 and contribute to eosinophil adhesion to the target vascular bed [9,10]. VCAM-1 binding to $\alpha_4\beta_1$ integrin also influences ICAM-1's adhesion to $\alpha_L\beta_2$ integrin [17] and both adhesion molecules are up-regulated in AC [11,14].

Studies with adhesion antagonists and knock-out mice further confirm their contribution to eosinophil recruitment in allergic inflammation [18]. These findings suggest that the blockade of $\alpha_4\beta_1$ might be put to good use in allergic eye disease and agree with numerous previous observations that this strategy is in fact useful in treating inflammatory disorders in animal models and humans [19,20]. Its direct role in basophil or mast cell recruitment has not been thoroughly investigated, but it appears that this integrin can influence their functional responses [18]. Mast cell activation is responsible for the clinical aspects of the early-phase reaction (EPR) triggered by a conjunctival allergen challenge, while the recruitment and activation of inflammatory cells sustains the late-phase reaction (LPR) [21].

In this study, we provide evidence that levocabastine, but not the first-generation antihistamine chlorpheniramine, can bind to the $\alpha_4\beta_1$ integrin and prevent eosinophil adhesion to VCAM-1, FN and to endothelial cells *in vitro*. Similarly, levocabastine prevented $\alpha_L\beta_2$/ICAM-1-mediated adhesion of Jurkat cells. In a scintillation proximity assay levocabastine, but not chlorpheniramine, displaced ^{125}I-FN binding to human $\alpha_4\beta_1$ integrin and, in flow cytometry analysis, levocabastine antagonized the binding of a primary antibody to integrin α_4 expressed on the Jurkat cell surface. In a model of AC levocabastine eye drops reduced the clinical aspects of the LPR and the conjunctival expression of $\alpha_4\beta_1$ integrin, by reducing infiltrated eosinophils. We propose that $\alpha_4\beta_1$ and $\alpha_L\beta_2$ integrin might be a cellular target of the antiallergic action of levocabastine, with a role in preventing eosinophil adhesion and infiltration in AC.

2. Materials and methods

2.1. Materials

Levocabastine hydrochloride and levocabastine 0.05% eye drops were provided by Bausch & Lomb (Montpellier, France). Trimeton injectable® (Schering-Plough Italia, Segrate, Milan; containing chlorpheniramine acetate) was purchased from a local pharmacy and diluted in saline for *in vivo* studies. Levocabastine hydrochloride was dissolved in propylene glycol/ethanol (1:1, w/w) and diluted at least 1:50 in Hank's balanced salt solution (HBSS; Cambrex, Profarmaco Milano, Milan, Italy) for *in vitro* studies. Vehicle was HBSS containing 2% of propylene glycol/ethanol (1:1, w/w). Cell culture media, HBSS and chloromethylfluorescein diacetate (CMFDA) were from Invitrogen (Carlsbad, CA, USA). Phosphate-buffered saline (PBS) and fetal bovine serum (FBS) were purchased from Cambrex. Lectin from *Triticus vulgaris*, soluble FN from human plasma, ovalbumin grade V, crystallized and lyophilized fraction, aluminum hydroxide, *o*-phenylenediamine, hydrogen peroxide (30%), Triton X-100, G418 and peroxidase acidic isoenzyme from horseradish were purchased from Sigma–Aldrich (Steinheim, Germany). Jurkat clone E6.1, HEK-293, EoL-1 and human umbilical vascular endothelial cells (HUVEC) were obtained from the European Cell Culture Collection (ECACC, Wiltshire, UK). Black 96-well clear-bottom plates were purchased from Corning Costar (Celbio, Milan, Italy). Soluble human VCAM-1 was purchased from R&D Systems (Minneapolis, MN, USA). BIO-1211 (*N*-[[4-[[[(2-methylphenyl)amino]carbonyl]amino]-phenyl]acetyl]-L-leucyl-L-aspartyl-L-valyl-L-proline) was purchased from Bachem (Weil am Rhein, Germany). Tissue Protein Extraction Reagent (T-PER®) and BCA® protein assay were purchased from Pierce (Rockford, IL, USA). Mouse anti-human and rabbit anti-human monoclonal antibodies against the α_4 subunit of $\alpha_4\beta_1$ integrin were purchased, respectively, from Calbiochem (Nottingham, UK) and Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Mouse anti-human monoclonal antibody against ICAM-1 was obtained from Chemicon International (Temecula, CA, USA). Secondary antibody (goat anti-mouse or goat anti-rabbit) and Cruz marker (sc-2035) were purchased from Santa Cruz Biotechnology Inc. Polyacrylamide gel, *N*,*N*,*N*′,*N*′-tetramethy-

lethylenediamine (TEMED), ammonium persulfate (PSA) and SDS were purchased from Sigma–Aldrich. Protran BA-85 (cellulose nitrate) was purchased from Schleicher & Schuell Italia (Legnano, Italy). Na[^{125}I] was obtained from Amersham Biosciences (GE Healthcare Europe, Milan, Italy). Polyvinyltoluene (PVT) anti-rabbit binding beads were supplied by Amersham Biosciences as a powder and reconstituted in distilled water. Plastic disposables were from Sarstedt (Verona, Italy). All other reagents were of analytical grade or the highest purity available, purchased from Sigma or Roche-Boehringer (Mannheim, Germany).

2.2. Animals

Male Dunkin-Hartley guinea pigs (250–300 g) were purchased from Charles-River (Calco, Italy). Animal procedures followed the guidelines of the Animal Care and Use Committee of the University of Bologna and conformed to the Association for Research in Vision and Ophthalmology (ARVO) resolution on the use of animals in research.

2.3. Cell culture

The human T cell line Jurkat (clone E6.1) was cultured in RPMI 1640 medium supplemented with L-glutamine, 10% heat-inactivated FBS, antibiotic-antimycotic solution in a humidified incubator at 37 °C with 5% CO_2. EoL-1 cells [22] were grown in RPMI-1640 with L-glutamine and 10% FBS in 75-cm^2 culture flasks and kept at 37 °C in a 5% CO_2 humidified atmosphere. HEK-293 cells, stably transfected with the human α_4 integrin cDNA (Origene, Rockville, MD, USA) cloned into pcDNA3.1 (Invitrogen, Milan, Italy), were cultured in minimum essential medium with L-glutamine, supplemented with 10% (v/v) FBS, non-essential amino acids and antibiotics, and kept at 37 °C in a humidified atmosphere (5% CO_2). Cells were transfected using a modified calcium phosphate procedure (Invitrogen) and positively transfected cells were selected with 400 μg/ml of G418 for at least 3 weeks.

HUVEC were grown in endothelial cell growth medium (ECACC) and used within six passages in all experiments. Human eosinophils, isolated from whole blood by density centrifugation followed by negative selection using immunomagnetic anti-CD16 beads (purity and viability were greater than 95%), were purchased from 3H Biomedical AB (Uppsala, Sweden) and cultured in completed cell culture medium (3H Biomedical) added of interleukin-5 (1 ng/ml; R&D Systems) and granulocyte macrophage-colony stimulating factor (10 ng/ml; R&D Systems) (for 24 h before to start adhesion assays).

2.4. Scintillation proximity-binding assay (SPA)

We developed a SPA assay to detect competitive binding of drugs to soluble ^{125}I-human FN (M_w approximately 440 kDa) bound to an antibody-captured integrin complex. The assay uses microspheres coated with an anti-rabbit IgG antibody capable of binding the complex $\alpha_4\beta_1$ integrin anti-α_4 antibody. The radioligand binds to the $\alpha_4\beta_1$ integrin and the close proximity of the isotope to the scintillant incorporated in the beads allows the radiation energy to transfer to the scintillant where it can be detected as counts per min (cpm). FN was labeled with Na[^{125}I] using the Bolton–Hunter reagent, as specified by the manufacturer. [^{125}I]-FN was purified from unincorporated iodine by gel filtration chromatography on PD-10 columns (GE Healthcare); trichloroacetic acid-precipitable radioactivity was 6.30×10^{10} μCi/mol. The experiments were carried out in scintillation vials; in each vial 1 mg/50 μl anti-rabbit-coated beads, 200 μg of rabbit anti-α_4 integrin antibody and a portion of cell eluate (containing approximately 100 μg of $\alpha_4\beta_1$ integrin) were added. The $\alpha_4\beta_1$ integrin was extracted from HEK-293 cells stably expressing the α_4 integrin subunit (these cells endogenously express the β_1 integrin subunit: data not shown). The cells were collected with a cell scraper then cytoplasmic proteins were extracted with the T-PER® extraction buffer and $\alpha_4\beta_1$ integrin was purified by affinity chromatography, as described [23]. For Western blot experiments on extracts we used antibodies against α_4 and β_1 integrin subunits to confirm that both integrins partitioned to the cell lysate collected from the affinity column. The binding buffer contained Tris–HCl 25 mM pH 7,5; $CaCl_2$ 1 mM; $MgCl_2$ 1 mM; $MnCl_2$ 1 mM; BSA 2% (w/v); phenylmethanesulfonyl fluoride (PMSF) 1 mM; aprotinin 1 mg/ml; leupeptin 50 mM.

First, we allowed for the slow interaction between the α_4 integrin protein and the rabbit anti-human α_4 integrin antibody by incubating them together for 1 h at 4 °C. Then the anti-rabbit antibody binding beads were added, and the solution containing the three components was incubated for 2 h at 4 °C in the dark. From this point on, all incubations were conducted at room temperature. [^{125}I]-FN was added to the vials, which were then incubated overnight on a shaker in the dark. Non-specific binding was determined in the presence of the specific $\alpha_4\beta_1$ integrin antagonist BIO-1211 (100 μM). The samples were read using a LS 6500 multipurpose scintillation counter (Beckham Coulter, Fullerton, CA, USA). The SPA procedure was optimized in preliminary experiments, as described [24].

2.5. Adhesion assays

Cell adhesion assays were done was as described [25]. Briefly, 96-well plates were coated at 4 °C overnight with 5 μg/ml of VCAM-1 or ICAM-1 and a saturation curve for the ligand was plotted to establish the best signal-to-noise ratio. Non-specific hydrophobic binding sites were blocked by incubation with a BSA (1%)/HBSS (w/v) solution for 30 min at 37 °C. The day of the assay, the cells were counted and stained with 12.5 μM CMFDA (30 min at 37 °C). After three rinses with BSA/HBSS to wash away the excess dye, aliquots of 50,000 (EoL-1) or 500,000 (Jurkat) cells were divided among a number of tubes corresponding to the number of treatments. For inhibition experiments, cells were mixed with the drug and pre-incubated at 37 °C for 30 min to reach equilibrium before being plated. After 30 min incubation at 37 °C in the coated wells, the non-specifically bound cells were washed away with BSA/HBSS solution. Adherent cells were lysed by the addition of 0.5% Triton X-100 in PBS (30 min at 4 °C). Released CMFDA was quantified by fluorescence imaging at Ex485 nm/Em535 nm (Wallac ARVO 1420 multilabel counter) and adherent cells were counted by interpolation on a standard curve. The fluorescence intensity with and without VCAM-1 or ICAM-1 was taken as, respectively, 100% and 0%. Alternatively, the number of adherent cells was calculated by comparison

with a standard curve prepared in the same plate using known concentrations of labeled cells. The efficacy of putative antagonists (at least eight different concentrations were used) was determined by the reduction in adherent cells compared to the untreated control.

Human eosinophil adherence was assessed as eosinophil peroxidase activity of adherent cells as described [26]. Briefly, 50 μl of eosinophils (4×10^5 cells/ml in BSA/HBSS) were placed in VCAM-1-coated wells and incubated for 30 min at 37 °C. After three washes with BSA/HBSS (37 °C) to remove non-adherent cells, 50 μl of BSA/HBSS was added to the reaction wells, while 50 μl of the original cell suspension was added to empty wells as a measure of total peroxidase. Eosinophil peroxidase substrate (1 mM hydrogen peroxide, 1 mM *o*-phenylenediamine HCl, and 0.1% Triton X-100 on Tris buffer, pH 8.0) was added to each well. After a 30-min incubation at room temperature, 25 μl of 4 M H_2SO_4 were added to each well to stop the reaction. Absorbance was measured at 490 nm in a microplate reader (Wallac ARVO 1420 multilabel counter). The detection of eosinophil peroxidase by this assay was linear between the concentrations of 10^2 and 3×10^4 eosinophil/well as determined by a standard curve. Adherence was calculated by comparing absorbance of unknowns to that of the standard curve.

Adhesion assays were also done in the presence of the mouse anti-human α_4 integrin antibody (5 μg/ml). Each experiment was conducted in quadruplicate and the data are presented as the mean ± S.E.M. of at least three independent assays.

HUVEC (2×10^4 cells/well) were grown to confluence in a 96-well plate and stimulated for 12 h with 25 ng/ml of TNF-α (R&D Systems) adopting a procedure already described [27], then co-cultured with CMFDA-labeled EoL-1 cells (1×10^5 cells/well) for 30 min at 37 °C in 5% CO_2. Levocabastine (100 or 400 μM) or the anti-α_4 integrin antibody (5 μg/ml) was added for 30 min to EoL-1 cells, 60 min before addition to HUVEC. After incubation, non-adhering EoL-1 cells were removed by gentle aspiration, and wells washed with PBS. Attached cells were lysed and the fluorescence intensity was determined as reported above.

2.6. Flow cytometry analysis (FACS)

FACS analysis was done to characterize levocabastine's specificity of action on the integrin receptors. Jurkat cells (10^6 per sample) were incubated with levocabastine (100 and 400 μM) at 37 °C for 30 min in BSA/HBSS; then all samples except the negative controls were incubated with a saturating concentration of the primary mouse anti-human α_4 integrin antibody for 30 min at 4 °C; subsequently, a fluorescein isothiocyanate (FITC)-conjugated secondary antibody was added for 45 min at 4 °C in the dark. After each incubation step, the cells were washed with BSA/HBSS. Finally, the cells were resuspended in HBSS/1% BSA and analyzed in an EPICS® ELITE (Beckman Coulter®, Milan, Italy) flow cytometer [28]. Non-specific fluorescence was evaluated by measuring the binding of FITC-conjugated secondary antibody in the absence of the primary antibody. The control binding of rabbit IgG (Calbiochem) was included.

Surface expression of α_4 integrin was examined in EoL-1 cells treated with levocabastine (100 and 400 μM) for 30 min, then incubated for 12 h in FN- (10 μg/ml) or BSA- (100 μg/ml) coated wells. Cells were washed with PBS and firmly adhering cells were incubated for 45 min at 4 °C with an anti-human α_4 integrin antibody, then for another 45 min with fluos-conjugated Annexin-V for the detection of phosphatidylserine (BD Biosciences, Erembodegem, Belgium) and propidium iodide to exclude apoptotic and necrotic cells [29]. After washing with PBS, cells were examined by FACS analysis. The mean fluorescence intensity was determined from at least 15,000 viable cells.

2.7. Active anaphylaxis in the guinea pig

Male Dunkin-Hartley guinea pigs were actively immunized by i.p. injection of 200 μg ovalbumin in 2 ml saline with 40 mg aluminum hydroxide (positive control), or saline alone (negative control), as described by Khosravi et al. [30]. Three weeks later, ovalbumin-treated guinea pigs were challenged with 30 μl per eye of saline solution, containing 100 mg/ml ovalbumin, instilled into the conjunctival sac. Levocabastine (0.05%) or chlorpheniramine (0.1%) solutions were instilled into the conjunctival sac (30 μl per eye, 60 and 30 min before ovalbumin); negative controls received saline alone.

Pictures of both eyes were taken for the clinical score before and then 1 and 6 h after the ovalbumin challenge. Conjunctival clinical symptoms were evaluated on both eyes according to the following scale: 0, no symptoms; 1, slight conjunctival redness with or without tears and minimal chemosis; 2, mild conjunctival redness with tears and moderate chemosis; 3, severe conjunctival redness with tears and partial lid eversion; 4, severe conjunctival redness with tears and lids more than half closed. Scoring was on a blind basis. The animals were euthanized 24 h after ovalbumin challenge by i.p. injection of Tanax® (3 ml/kg; Hoechst AG, Frankfurt-am-Main, Germany) and the conjunctivas were collected for the subsequent investigations.

2.8. Eosinophil peroxidase assay

Eosinophil peroxidase assay was done as previously described [31]: 24 h after induction of conjunctivitis by conjunctival challenge with the antigen, the guinea pigs were euthanized and the upper and lower parts of the conjunctiva were excised, cleaned, weighed and washed twice with ice-cold PBS. The tissues were homogenized with 50 mM Tris–HCl buffer (pH 8.0) using a Potter-Elvejehm glass/Teflon homogenizer (Wheaton, Millville, NJ, USA) on ice. After addition of 350 μl of 50 mM Tris–HCl buffer and 150 μl of 0.1% Triton X-100. The homogenates were placed in an ice bath for 1 h. Then the substrate solution (400 μl of 50 mM Tris–HCl buffer containing 0.1% Triton X-100, 1 mM *o*-phenylenediamine, and 0.5 mM hydrogen peroxide) was added to 200 μl of the sample and incubated at 37 °C for 10 min. The reaction was stopped with 200 μl of 4 M H_2SO_4. Absorbance was measured using a spectrophotometer (JASCO V-530, Jasco, UK) at 490 nm.

A standard curve was plotted with different concentrations of peroxidase diluted in 50 mM Tris–HCl buffer (pH 6.0) containing 1 mM *o*-phenylenediamine, and 0.5 mM hydrogen peroxide. Eosinophil peroxidase activity was measured according to the method of Strath et al. [32], which is based on the

oxidation of *o*-phenylenediamine by eosinophil peroxidase in the presence of hydrogen peroxide. One unit corresponds to 1 mmol of hydrogen peroxide decomposed in 10 min, and the results were expressed as eosinophil peroxidase levels (mU of enzyme/mg wet tissue).

2.9. Western blotting

Conjunctivas from immunized and non-immunized animals were homogenized in T-PER® reagent (Pierce) and proteins were extracted following the manufacturer's protocol. The protein content was quantified using a BCA protein assay. Proteins from the cytoplasmic fraction (150 μg) were denatured at 99 °C for 4 min before being loaded and separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). We used the Cruz marker sc-2035 as molecular weight standard. Proteins were transferred to Protran membranes which were blocked with 5% non-fat milk in TBS (10 mm Tris–HCl, pH 8, containing 150 mm NaCl) plus 0.1% Tween 20 for 1.5 h at 25 °C. The blots were then probed for 1.5 h at 25 °C in TBS containing 0.1% Tween 20, 5% non-fat milk and antibodies with a dilution of 1:800 for α_4 integrin monoclonal antibody, and 1:5000 for β-actin antibody (used as loading control for cytoplasmic lysates). The membranes were incubated with peroxidase-conjugated secondary antibodies at a 1:10,000 dilution. Blots were developed with SuperSignal West Pico chemiluminescent substrate according to the manufacturer's protocol (Pierce). Chemiluminescence was acquired using a luminescent image analyzer LAS-3000 (Fuji-Film). An image AIDA (Advanced Image Data Analyzer) software (Raytest) was used to quantify the intensity of the bands.

2.10. Data analysis

All data are expressed as mean ± S.E.M., for the number of experiments indicated. Statistical comparisons were made by ANOVA and post hoc Newman-Keuls test with differences of $P < 0.05$ considered significant.

For the clinical score, each group comprised five animals. Non-parametric analysis of the scores assigned to the conjunctival symptoms was conducted using the Friedman test followed by Dunn's post hoc comparison test. IC_{50} indicates the molar drug concentration required to inhibit the response by 50%. Data were analyzed using GraphPad software (GraphPad Software Inc., San Diego, CA, USA).

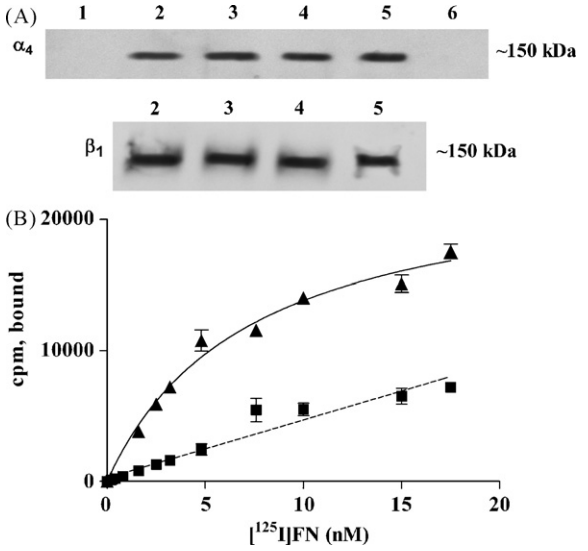

Fig. 1 – Levocabastine binds to the antibody-captured $\alpha_4\beta_1$ integrin in the SPA. Panel A, Representative autoradiogram of Western blot experiments, described under Section 2, to evaluate the α_4 and β_1 integrin subunits in cell lysates of HEK-293 cells stably expressing α_4 integrin. The lysates were purified by affinity chromatography and fractions of approximately 1 ml were collected; the presence of the α_4 or β_1 integrin subunit was evaluated on aliquots of 50 μl by Western blotting. Six fractions were collected and the presence of α_4 integrin was confirmed in all except the first (void elution volume) and the last. The fractions with the α_4 integrin subunit were then assayed to confirm the presence of the β_1 integrin subunit (fractions 2–5). Approximate molecular masses of the α_4 and β_1 integrin subunits [see ref. [51]] were determined by comparison with molecular mass standards. Fractions 2–5 were used for SPA. Panel B, Saturation SPA binding of [^{125}I]-FN to the purified $\alpha_4\beta_1$ integrin extracted from HEK-293 cells stably expressing α_4 integrin and incubated with increasing concentrations of the radioligand (cpm) as described under Section 2. Non-specific binding was determined in the presence of 100 μM unlabeled BIO-1211. (■) Non-specific binding; (▲) specific binding obtained by subtraction of the non-specific binding from total binding counts. Mean ± S.E.M. of three experiments done in triplicate.

3. Results

3.1. Binding affinity of levocabastine to human $\alpha_4\beta_1$ integrin by SPA

HEK-293 cells stably expressing human α_4 integrin were used to measure levocabastine binding to $\alpha_4\beta_1$ integrin by SPA. Western blot analysis of the $\alpha_4\beta_1$ integrin, extracted from cell lysates and purified by a chromatographic technique already reported [23], confirmed that both α_4 and β_1 integrin subunits were present in the eluate employed for the SPA (Fig. 1, panel A). Specific binding of ^{125}I-FN to an antibody-captured $\alpha_4\beta_1$ integrin was time-dependent; the signal increasing during the first 10 h, then reaching a plateau and remaining constant for the remainder of the 24 h incubation (data not shown). The relatively slow kinetics of the SPA may require the establishment of equilibrium between the different components [33]. ^{125}I-FN specific binding, measured after overnight incubation, was concentration-dependent (Fig. 1, panel B) and was blocked in the presence of 100 μM BIO-1211 (a potent $\alpha_4\beta_1$ and $\alpha_1\beta_2$ integrin antagonist) or an anti-human α_4 integrin antibody (5 μg/tube) to capture the integrin complex (data not shown). Levocabastine caused concentration-dependent inhibition of ^{125}I-FN binding to the SPA bead-associated $\alpha_4\beta_1$ integrin (Fig. 2) with an IC_{50} of 406.2 μM.

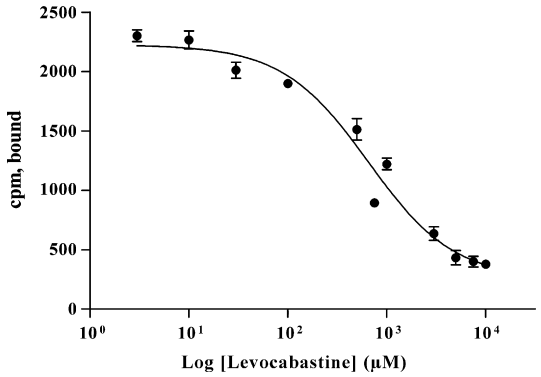

Fig. 2 – Inhibition of [^{125}I]-FN binding by levocabastine in the SPA. Binding to HEK-293 cell lysates was measured in the presence of increasing concentrations of levocabastine (0.003–10 mM) and of a fixed amount of [^{125}I]-FN (100,000 cpm) as described under Section 2. Mean ± S.E.M. of three experiments done in triplicate.

3.2. *Levocabastine competes with a specific antibody for binding to α_4 integrin in Jurkat cells*

FACS analysis confirmed that levocabastine binds to α_4 integrin (Fig. 3): at 100 and 400 μM caused concentration-related inhibition of the binding of the primary antibody to α_4 integrin expressed on the Jurkat cell surface.

3.3. *Levocabastine inhibits $\alpha_4\beta_1$ integrin/VCAM-1-mediated cell adhesion in vitro*

The human Jurkat and EoL-1 cell lines express $\alpha_4\beta_1$ integrin on their surface [22,34]. In a first series of experiments, we ascertained that adhesion of these cells to 96-well plates coated with human recombinant VCAM-1 or FN was concentration-dependent and was not observed in BSA-coated wells. The adhesion of Jurkat cells (5×10^5 cells were added to each well) to VCAM-1 (2–25 μg/ml) ranged from 1.9 to 3.2×10^4 cells and the adhesion to FN (2–25 μg/ml) ranged from 1.6 to 4.3×10^4 cells. The adhesion of EoL-1 cells (5×10^4 cells were added to each well) ranged from 1.3 to 2.5×10^4 cells and the adhesion to FN (2–25 μg/ml) ranged from 1.1 to 2.1×10^4 cells. Finally, we ascertained that the adhesion of human eosinophils (2×10^4 cells were added to each well) to VCAM-1 (2–25 μg/ml) ranged from 0.9 to 2.2×10^4 cells. The adhesion of Jurkat, EoL-1 and human eosinophil cells to VCAM-1 (10 μg/ml) was inhibited by more than 92% after pretreatment with 5 μg/ml anti-α_4 integrin antibody and adhesion of Jurkat or EoL-1 cells to FN (10 μg/ml) was inhibited by 92% in the presence of this antibody. Neither drug (upto 2 mM) influenced cell viability, evaluated by Trypan blue exclusion (data not shown). In the light of these results, for adhesion assays we coated the well plates with 10 μg/ml of VCAM-1 or FN.

As shown in Table 1, levocabastine inhibited $\alpha_4\beta_1$ integrin-dependent adhesion of Jurkat cells to VCAM-1 with an IC_{50} of 395.6 μM, and the adhesion of EoL-1 cells with an IC_{50} of 403.6 μM. Moreover, levocabastine inhibited adhesion of human eosinophils to VCAM-1-coated wells (IC_{50} = 443.7 μM). Levocabastine displayed a similar activity to inhibit $\alpha_4\beta_1$ integrin-dependent adhesion of Jurkat and EoL-1 cells to FN and also prevented $\alpha_L\beta_2$ integrin-dependent adhesion of Jurkat cells to ICAM-1 (IC_{50} = 33.3 μM). In contrast, chlorpheniramine, a potent blocker of H_1 receptors [35], did not affect Jurkat cell adhesion to VCAM-1, FN and ICAM-1 (Table 1).

Finally, we ascertained that, in these experimental conditions, the small peptide BIO-1211 behaves as a potent $\alpha_4\beta_1$ and $\alpha_L\beta_2$ integrin antagonist and inhibited Jurkat cell adhesion to VCAM-1-, FN- or ICAM-1-coated wells (Table 1).

Exposure of endothelial cells to the inflammatory cytokine TNF-α increases the surface expression of cell adhesion

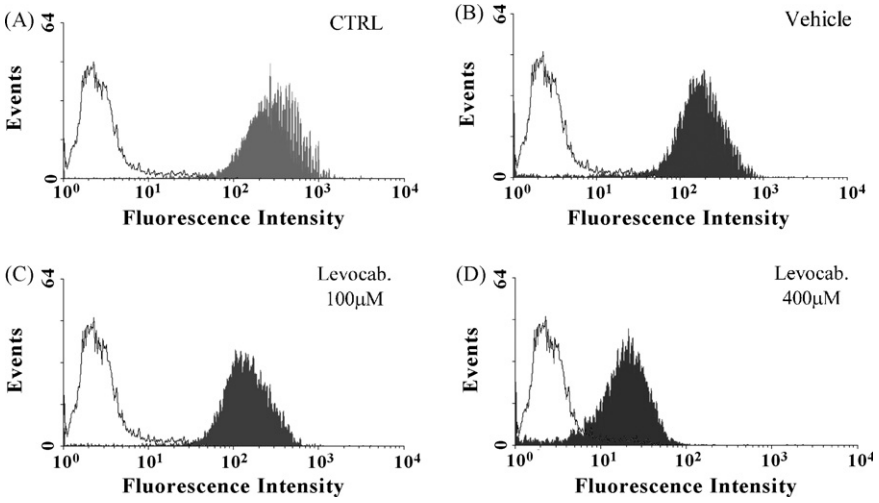

Fig. 3 – Levocabastine competes with a specific antibody for binding to α_4 integrin in Jurkat cells. Cells were incubated with an anti-human α_4 integrin antibody (5 μg/tube) in the absence (Ctrl; panel A) or presence of 100 μM levocabastine (C) or 400 μM levocabastine (D) or in the presence of the vehicle alone (B). After washing, 10 μg/ml FITC-conjugated goat anti-rabbit IgG was added and the samples were incubated for 45 min at 4 °C in the dark as described under Section 2. After washing out the excess of unbound secondary antibody, the fluorescence was measured by flow cytometry. The control binding of rabbit IgG is shown in the unfilled trace.

Table 1 – Effects of levocabastine, BIO-1211 and chlorpheniramine on different integrin-mediated human cell adhesion systems

Integrin-mediated cell adhesion	IC_{50} (μM)
Levocabastine	
$\alpha_4\beta_1$-mediated cell adhesion	
Jurkat/VCAM-1	395.6 (300.8–520.1)[a]
EoL-1/VCAM-1	403.9 (349.0–543.5)
Human eosinophils/VCAM-1	443.7 (366.0–568.9)
Jurkat/FN	350.7 (315.2–532.3)
EoL-1/FN	360.6 (325.7–477.6)
$\alpha_L\beta_2$-mediated cell adhesion	
Jurkat/ICAM-1	33.3 (14.7–75.5)
BIO-1211	
$\alpha_4\beta_1$-mediated cell adhesion	
Jurkat/VCAM-1	8.6×10^{-3} ($4.5–10.1 \times 10^{-3}$)
EoL-1/VCAM-1	7.2×10^{-3} ($3.9–9.7 \times 10^{-3}$)
Jurkat/FN	7.5×10^{-3} ($4.2–10.4 \times 10^{-3}$)
EoL-1/FN	6.6×10^{-3} ($5.2–9.2 \times 10^{-3}$)
$\alpha_L\beta_2$-mediated cell adhesion	
Jurkat/ICAM-1	8.4×10^{-5} ($4.7–14.8 \times 10^{-5}$)
Clorpheniramine	
$\alpha_4\beta_1$-mediated cell adhesion	
Jurkat/VCAM-1	N.E.[b]
Jurkat/FN	N.E.
$\alpha_L\beta_2$-mediated cell adhesion	
Jurkat/ICAM-1	N.E.

Three independent experiments were run in quadruplicate and IC_{50} were calculated as described under Section 2.
[a] 95% Confidence limits.
[b] N.E., No effect upto 2 mM.

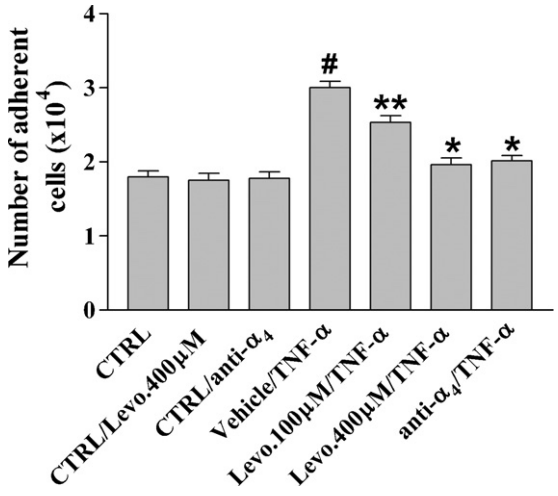

Fig. 4 – Levocabastine and an anti-α_4 integrin antibody reduce TNF-α-induced EoL-1 binding to HUVEC. HUVEC were treated for 12 h with TNF-α (25 ng/ml) dissolved in HBSS or with HBSS alone (CTRL). EoL-1 cells exposed for 1 h to the vehicle (HBSS containing 2% of propylene glycol/ethanol), levocabastine (100 and 400 μM) or an anti-α_4 integrin antibody (5 μg/ml) and treated with CMFDA were co-cultured with HUVEC for 30 min. In CTRL cells, levocabastine or an anti-α_4 integrin antibody did not influence EoL-1-cell adhesion. Firmly adhering EoL-1 cells were counted as described under Section 2. Mean ± S.E.M. from four separate experiments carried out in triplicate. $^{\#}P < 0.001$ compared to CTRL. $^{**}P < 0.01$; $^{*}P < 0.001$ compared to vehicle/TNF-α-stimulated HUVEC (Newman-Keuls test after ANOVA).

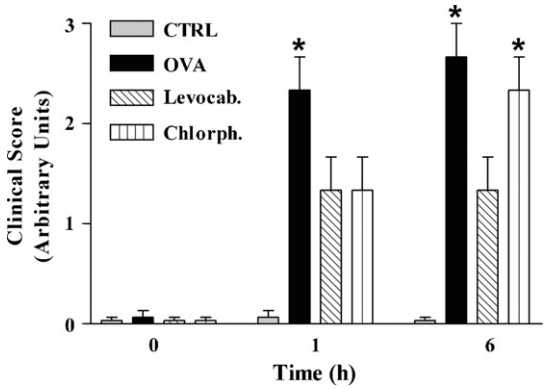

Fig. 5 – Effects of levocabastine and chlorpheniramine on conjunctival symptoms induced by ovalbumin in guinea pigs actively immunized by i.p. injection of ovalbumin and, 2 weeks later, challenged with ovalbumin (30 μl of 2.5% solution) instilled into both eyes. Levocabastine (0.05%), and chlorpheniramine (0.1%) were instilled into both eyes (30 μl per eye) 60 and 30 min before ovalbumin challenge; controls received the vehicle alone and were not treated with ovalbumin. Each group comprised five guinea pigs and the score was based on changes before and then 1 and 6 h after challenge for the eye symptoms of itching, swelling, redness, and lid eversion, as described under Section 2. Mean ± S.E.M. ($n = 5$). $^{*}P < 0.01$ vs. vehicle-treated group (Friedman test followed by Dunn's post hoc comparison).

molecules [9]. Kwon et al. [27] reported that HUVEC do not express VCAM-1 in the resting state, but the expression of this cell adhesion molecule is increased after exposure to TNF-α. Previously, it has been reported that eosinophils adhere spontaneously to HUVEC [36,37]. In agreement with these observations, EoL-1 cells adhered spontaneously to untreated HUVEC (Fig. 4). Basal adhesion of EoL-1 to HUVEC (approximately 18% of the added cells) was not significantly affected by levocabastine (400 μM) or an anti-α_4 integrin antibody (Fig. 4). Therefore, we investigated whether levocabastine influenced EoL-1 adhesion to TNF-α-stimulated HUVEC. As shown in Fig. 4, the number of EoL-1 cells adherent to HUVEC significantly increased after 12 h exposure to TNF-α (25 ng/ml). Levocabastine (100 and 400 μM) reduced this increment, in a concentration-dependent manner, as did an anti-α_4 integrin antibody.

3.4. *Levocabastine did not influence FN-induced α_4 integrin expression in EoL-1 cells*

Adhesion molecules play an important role in eosinophilic activation and in adhesion to extracellular matrix and accumulation at inflamed sites since cell recruitment is dependent on both expression and functional activity of these molecules [9]. In eosinophils, the interaction of $\alpha_4\beta_1$ integrin with FN upregulated the expression of ICAM-1 [38]. As regards $\alpha_4\beta_1$ integrin, employing FACS analysis, we found that after 12 h

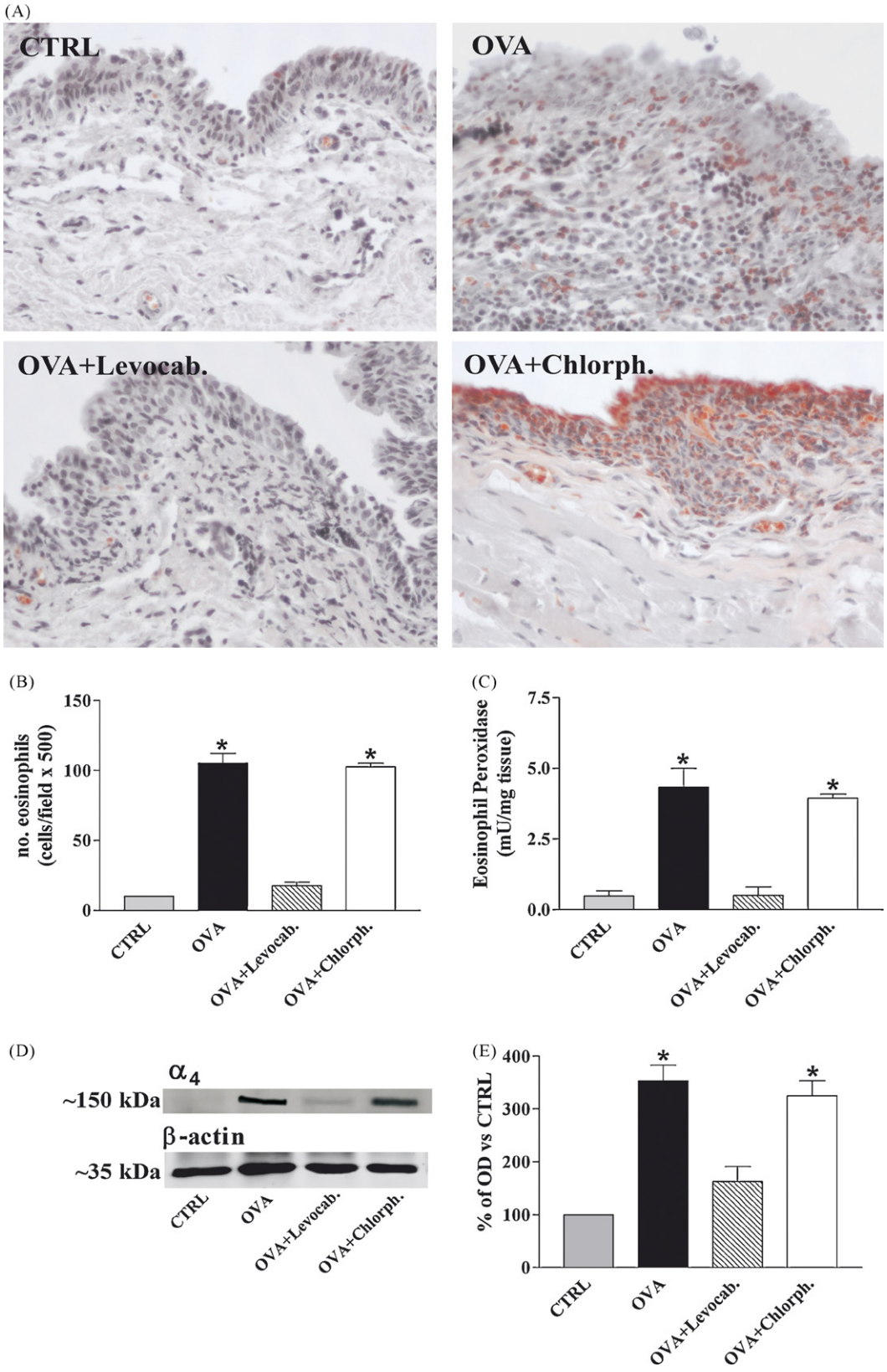

Fig. 6 – Panel A, Photomicrographs of the conjunctiva 24 h after topical challenge with ovalbumin. Substantial eosinophil infiltration is observed in ovalbumin-treated guinea pigs (OVA) in comparison to negative controls (animals not immunized with ovalbumin) who received saline alone and were not challenged with ovalbumin (CTRL). In levocabastine-treated guinea pigs (Levocab.) there was much less eosinophil infiltration than in the ovalbumin-treated group and in chlorpheniramine-treated animals (Chlorph.). Panel B, Effects of levocabastine and chlorpheniramine on eosinophil infiltration in the guinea pig conjunctiva 24 h after topical challenge with ovalbumin. The eosinophils in each field

adhesion of EoL-1 cells to FN-coated plates the expression of this integrin was not modified in comparison to that observed in cells added to BSA-coated plates, as ascertained by evaluating mean fluorescence intensity (MFI) from 15,000 cells. Pretreatment with levocabastine (100 and 400 μM) did not down-regulate the profile of $\alpha_4\beta_1$ integrin in EoL-cells added to FN- or BSA-coated plates (data not shown).

3.5. *Effect of levocabastine on conjunctival symptoms and conjunctival eosinophil recruitment in ovalbumin-sensitized guinea pigs*

Guinea pigs were actively immunized by i.p. injection of ovalbumin and 2 weeks later challenged with ovalbumin instilled into the conjunctival sac. One hour after challenge, during the ocular EPR to ovalbumin, swelling of the eyelids and chemosis were more marked in treated animals than controls, but was significantly reduced by 0.05% levocabastine and 0.1% chlorpheniramine eye-drops before treatment (30 μl per eye 60 and 30 min before ovalbumin) (Fig. 5). However, only levocabastine-pretreated guinea pigs showed a significant reduction in the severity of conjunctival symptoms during the LPR of AC 6 h after ovalbumin challenge.

The guinea pigs were euthanized 24 h later and histological analysis showed numerous eosinophils infiltrating the conjunctiva of ovalbumin-treated animals. The eosinophil infiltration was much less marked in levocabastine-treated guinea pigs than controls, and chlorpheniramine was ineffective (Fig. 6, panels A and B). Similarly, eosinophil peroxidase activity, taken as an index of eosinophil infiltration, increased 24 h after antigen challenge in control guinea pigs, whereas there was a noteworthy reduction in levocabastine-treated guinea pigs, but not in chlorpheniramine-treated animals (Fig. 6, panel C).

3.6. *Effect of levocabastine on integrin α_4 expressed in the conjunctiva of actively immunized guinea pigs*

Exposure of actively immunized guinea-pigs to the topical challenge of ovalbumin instilled into the conjunctival sac induced a significant increase, 24 h later, in α_4 integrin levels evaluated in conjunctival homogenates by Western blot analysis (Fig. 6, panels D and E). This increase was related to eosinophils and possibly other leukocytes infiltrating the conjunctiva and was significantly reduced by pretreatment with levocabastine eye-drops but not by chlorpheniramine.

4. Discussion

In *in vitro* experiments, we obtained evidence that levocabastine, but not the first-generation antihistamine chlorpheniramine, binds to the $\alpha_4\beta_1$ integrin and prevents eosinophil adhesion to VCAM-1, FN or to HUVEC cells. These latter express VCAM-1 on their surface after TNF-α exposure [39]. SPA indicated that only levocabastine displaced ^{125}I-FN binding to human $\alpha_4\beta_1$ integrin and, in flow cytometry analysis, it antagonized the binding of a primary antibody to α_4 integrin expressed on the Jurkat cell surface. Similarly, levocabastine prevented $\alpha_L\beta_2$/ICAM-1-mediated adhesion of Jurkat cells [40]. Finally, in a model of AC levocabastine eye-drops reduced the clinical aspects of the LPR and the conjunctival expression of $\alpha_4\beta_1$ integrin by reducing infiltrated eosinophils.

The $\alpha_4\beta_1$ integrin/VCAM-1 pathway is crucial for the firm adhesion and transmigration of eosinophils into the conjunctiva through vascular endothelial cells. The inhibition of conjunctival eosinophil infiltration by an integrin α_4 monoclonal antibody has been reported in a guinea pig model of AC [15]. Treatment with anti-α_4 integrin and anti-VCAM-1 antibodies significantly suppressed the conjunctival eosinophil infiltration induced in mice by active immunization with ragweed, or adoptive transfer of ragweed-primed splenocytes [16]. Whitcup et al. [41] showed that antibodies against $\alpha_L\beta_2$ integrin and its counter-receptor ICAM-1 relieve both the clinical signs of immediate hypersensitivity and cellular infiltration into the conjunctiva, 24 h after antigen challenge in mice. Several antiallergic drugs, including levocabastine, down-regulate ICAM-1 expression [4–8]; Pesce et al. [42] reported that in patients with AC, conjunctival epithelial cells expressed $\alpha_L\beta_2$. Therefore, blocking $\alpha_4\beta_1$ and $\alpha_L\beta_2$ integrin-mediated cell adhesion may offer another strategy for the treatment of AC, as it prevents the development of the EPR and LPR.

$\alpha_4\beta_1$ and $\alpha_L\beta_2$ integrins may act in concert to mediate eosinophil adhesion and signaling functions to meet the peculiar challenges these cells face as they contact the vascular epithelium. ''Cross-talk'' between these integrins was proposed by Chan et al. [17] who suggested that $\alpha_4\beta_1$ integrin binding to VCAM-1 might also strengthen $\alpha_L\beta_2$-mediated adhesion of T lymphocytes to ICAM-1. As regards eosinophils, Higashimoto et al. [38] reported that adherence of EoL-1 to FN was mediated by $\alpha_4\beta_1$ integrin and enhanced ICAM-1 expression, and this might contribute to increasing cell adhesion to the extracellular matrix and conjunctival eosinophil accumulation.

(×500 magnification) were counted 24 h after antigen exposure. Controls received saline alone and were not challenged with ovalbumin (CTRL). Panel C, Effects of levocabastine and chlorpheniramine on conjunctival eosinophil peroxidase levels in the conjunctiva 24 h after topical challenge with ovalbumin. Controls received saline alone and were not challenged with ovalbumin (CTRL). Panels D and E, Effects of levocabastine and chlorpheniramine on conjunctival levels of α_4 integrin subunit 24 h after topical challenge with ovalbumin. Guinea pigs were euthanized, the conjunctiva was collected and protein extracted and used for Western blot analysis. A representative Western blot, repeated three times with similar results, and the densitometric analysis of the bands is shown. The approximate molecular mass of α_4 integrin was determined by comparison with molecular mass standards. OD refers to the relative optical density of each band, defined by normalization of the α_4 integrin band to the β-actin band (arbitrary units). A total of 150 μg of protein extract was loaded and separated in polyacrylamide gel, as described under Section 2. Mean ± S.E.M. (n = 5). $^*P < 0.01$ vs. CTRL (Newman-Keuls test after ANOVA).

Our data support this latter idea, as the blockade of eosinophil adhesion by levocabastine may prevent further eosinophil functional changes, including expression of adhesion molecules, such as ICAM-1 whereas levocabastine does not influence α_4 integrin expression. In fact, we observed that in the eosinophilic EoL-1 cell line levocabastine did not affect α_4 integrin expression in cells maintained for 12 h in FN- or BSA-coated wells. In agreement with our data, Fukuishi et al. [43] reported that the antiallergic drug olopatadine inhibited antigen-induced eosinophil infiltration and repressed interleukin-5-induced expression of $\alpha_L\beta_2$ integrin but not of $\alpha_4\beta_1$ integrin. These *in vitro* data may help explain the reduction of the expression of $\alpha_4\beta_1$ integrin that we observed in the conjunctiva of ovalbumin-sensitized guinea pigs treated with levocabastine. In fact, this effect might be a consequence of the reduction in recruitment and migration of eosinophils to the site of allergic inflammation by levocabastine rather than of levocabastine-induced $\alpha_4\beta_1$ down-regulation. Thus, hypothetically, levocabastine might exert its inhibitory effect on eosinophil adhesion binding to $\alpha_4\beta_1$ integrin, affecting its avidity as well as affinity for VCAM-1 and FN. In fact, integrins increase their affinity or avidity to endothelial ligands upon exposure to chemokines and can undergo to conformational changes upon ligand binding apart from expression changes on the leukocyte surfaces [9].

We confirmed the positive effect of levocabastine eye-drops on EPR and LPR inflammatory changes induced by allergen-specific conjunctival challenge. Histamine and eicosanoids are responsible for the typical EPR [44]. However, mast cells also contribute to the synthesis and release of cytokines, chemokines and growth factors, triggering a cascade of inflammatory events on the surface of epithelial and endothelial cells that leads to the LPR, with recruitment of eosinophils and neutrophils [45]. Inflammatory cytokines may enhance the expression of E-selectin, ICAM-1 and VCAM-1 on the vascular endothelial cells and initiate the rolling of immune cells [9,13,14].

According to previous studies, levocabastine's effect on EPR seems to be mediated by the blockade of histamine H_1 receptors and mast cell stabilization [1,2]. It reduces conjunctival eosinophil infiltration in the LPR and, as proved in this study, affects $\alpha_4\beta_1$ integrin functions. However, it cannot be ruled out that levocabastine reduces inflammatory cell infiltration in the LPR by affecting the release of cytokines and chemokines [3,46].

Conjunctival epithelial cells do not express $\alpha_4\beta_1$ integrin [47] and its occurrence in the conjunctiva could be a consequence of eosinophil infiltration promoted by different mechanisms, independent of histamine binding to H_1 receptors. This idea is further supported by experiments with the classical H_1 antagonist chlorpheniramine. In fact, a 0.1% chlorpheniramine eye-drops blocked only the EPR of AC and did not reduce eosinophil infiltration or expression of the adhesion molecule $\alpha_4\beta_1$ in the conjunctiva of guinea pigs treated with ovalbumin. This drug also did not modify Jurkat or EoL-1 cell adhesion to VCAM-1 or FN. First-generation antihistamines like chlorpheniramine are weak inhibitors of histamine-stimulated cytokine synthesis [3] while levocabastine is more potent [1,2]. Furthermore, chlorpheniramine, in an experimental model of allergic cutaneous antigen-induced inflammatory cell infiltration, did not significantly inhibit inflammatory cell infiltration in the LPR [48].

Levocabastine caused concentration-related blockade of adhesion of the EoL-1 cells to VCAM-1 and FN. The drug's potency *in vitro* (IC_{50} around 400 μM) is important when this molar concentration is converted to a percentage weight/volume (400 μM equals about 200 μg/ml and is equivalent to a 0.02% solution). Because the conjunctiva is the target tissue for this compound, penetration and transport into the eye do not significantly affect drug delivery. For these reasons, and in view of the clinical efficacy of 0.05% levocabastine in AC models [1], the effect on $\alpha_4\beta_1$ and $\alpha_L\beta_2$ integrin blockade appears acceptable. Levocabastine shows potent and sustained antiallergic activity after conjunctival administration. In ovalbumin-sensitized guinea pigs, the drug reduced or completely prevented conjunctival symptoms and this effect lasted upto 24 h [49]. Complete inhibition of histamine-induced conjunctivitis persisted 24 h after topical levocabastine [50]. Therefore, topical levocabastine may persist at an adequate conjunctival concentration for upto 6–8 h, long enough to interfere with eosinophil migration to the ocular surface.

We propose that levocabastine acts through different mechanisms as an antiallergic agent and some of the drug's cellular targets may play a role in eosinophil adhesion. Topical levocabastine, by blocking integrin-mediated cell adhesion, may inhibit the development of the late-phase of AC. This compound easily reaches conjunctival cells and vessels when administered topically, and can prevent the recruitment and activation of eosinophils as well as other immune cells (e.g. neutrophils and other leukocytes).

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REFERENCES

[1] Dechant KL, Goa KL. Levocabastine. A review of its pharmacological properties and therapeutic potential as a topical antihistamine in allergic rhinitis and conjunctivitis. Drugs 1991;41:202–24.
[2] Awouters F, Niemegeers CJ, Jansen T, Megens AA, Janssen PA. Levocabastine: pharmacological profile of a highly effective inhibitor of allergic reactions. Agents Actions 1992;35:12–8.
[3] Yanni JM, Weimer LK, Sharif NA, Xu SX, Gamache DA, Spellman JM. Inhibition of histamine-induced human conjunctival epithelial cell responses by ocular allergy drugs. Arch Ophthalmol 1999;117:643–7.
[4] Bielory L, Lien KW, Bigelsen S. Efficacy and tolerability of newer antihistamines in the treatment of allergic conjunctivitis. Drugs 2005;65:215–28.
[5] Buscaglia S, Paolieri F, Catrullo A, Fiorino N, Riccio AM, Pesce G, et al. Topical ocular levocabastine reduces ICAM-1 expression on epithelial cells both in vivo and in vitro. Clin Exp Allergy 1996;26:1188–96.

[6] Ciprandi G, Tosca MA, Cosentino C, Riccio AM, Passalacqua G, Canonica GW. Effects of fexofenadine and other antihistamines on components of the allergic response: adhesion molecules. J Allergy Clin Immunol 2003;112: S78–82.
[7] Schultz BL. Pharmacology of ocular allergy. Curr Opin Allergy Clin Immunol 2006;6:383–9.
[8] Ciprandi G, Pronzato C, Passalacqua G, Ricca V, Grogen J, Mela GS, et al. Topical azelastine reduces eosinophil activation and intercellular adhesion molecule-1 expression on nasal epithelial cells: an antiallergic activity. J Allergy Clin Immunol 1996;98:1088–96.
[9] Broide D, Sriramarao P. Eosinophil trafficking to sites of allergic inflammation. Immunol Rev 2001;179:163–72.
[10] Rose DM, Alon R, Ginsberg MH. Integrin modulation and signaling in leukocyte adhesion and migration. Immunol Rev 2007;218:126–34.
[11] Ciprandi G, Buscaglia S, Pesce G, Villaggio B, Bagnasco M, Canonica GW. Allergic subjects express intercellular adhesion molecule-1 (ICAM-1 or CD54) on epithelial cells of conjunctiva after allergen challenge. J Allergy Clin Immunol 1993;91:783–92.
[12] Elices MJ, Osborn L, Takada Y, Crouse C, Luhowskyj S, Hemler ME, et al. VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. Cell 1990;60: 577–84.
[13] Abu El-Asrar AMA, Geboes K, Al-Kharashi S, Tabbara KF, Missotten L, Desmet V. Adhesion molecules in vernal keratoconjunctivitis. Br J Ophtalmol 1997;81:1099–106.
[14] Bacon AS, McGill JI, Anderson DF, Baddeley S, Lightman SL, Holgate ST. Adhesion molecules and relationship to leukocyte levels in allergic eye disease. Invest Ophthalmol Vis Sci 1998;39:322–30.
[15] Ebihara N, Yokoyama T, Kimura T, Nakayasu K, Okumura K, Kanai A, et al. Anti VLA4 monoclonal antibody inhibits eosinophil infiltration in allergic conjunctivitis model of guinea pig. Curr Eye Res 1998;19:20–5.
[16] Fukushima A, Yamaguchi T, Ishida W, Fukata K, Ueno H. Role of VLA-4 in the development of allergic conjunctivitis in mice. Mol Vis 2006;12:310–7.
[17] Chan JR, Hyduk SJ, Cybulsky MI. Alpha 4 beta 1 integrin/VCAM-1 interaction activates alpha L beta 2 integrin-mediated adhesion to ICAM-1 in human T cells. J Immunol 2000;164:746–53.
[18] Bochner BS, Schleimer RP. Mast cells, basophils, and eosinophils: distinct but overlapping pathways for recruitment. Immunol Rev 2001;179:5–15.
[19] Teixeira MM, Robinson MK, Shock A, Hellewell PG. α_4 Integrin-dependent eosinophil recruitment in allergic but not non-allergic inflammation. Br J Pharmacol 2001;132:596–604.
[20] Yang GX, Hagmann WK. VLA-4 antagonists: potent inhibitors of lymphocyte migration. Med Res Rev 2003;23:369–92.
[21] Bonini S, Magrini L, Rotiroti G, Lambiase A, Tomassini M, Rumi C, et al. The eosinophil and the eye. Allergy 1997;52(Suppl. 34):44–7.
[22] Mayumi M. EoL-1, a human eosinophilic cell line. Leuk Lymphoma 1992;7:243–50.
[23] Solorzano C, Bouquelet S, Pereyra MA, Blanco-Favela F, Slomianny MC, Chavez R, et al. Isolation and characterization of the potential receptor for wheat germ agglutinin from human neutrophils. Glycoconj J 2006;23:591–8.
[24] Sun S, Almaden J, Carlson TJ, Barker J, Gehring MR. Assay development and data analysis of receptor-ligand binding based on scintillation proximity assay. Metab Eng 2005;7:38–44.
[25] Marcinkiewicz C, Calvete JJ, Marcinkiewicz MM, Raida M, Vijay-Kumar S, Huang Z, et al. EC3, a novel heterodimeric disintegrin from Echis carinatus venom, inhibits α_4 and α_5 integrins in an RGD-independent manner. J Biol Chem 1999;274:12468–73.
[26] Conran N, Ferreira HHA, Lorand-Metze I, Thomazzi SM, Antunes E, de Nucci G. Nitric oxide regulates human eosinophil adhesion mechanisms *in vitro* by changing integrin expression and activity on the eosinophil cell surface. Br J Pharmacol 2001;134:632–8.
[27] Kwon BC, Sohn MH, Kim KW, Kim ES, Kim KE, Shin MH. House dust mite induces expression of intracellular adhesion molecule-1 in Eol-1 human eosinophilic leukemic cells. J Korean Med Sci 2007;22:815–9.
[28] Marcinkiewicz C, Rosenthal LA, Mosser DM, Kunicki TJ, Niewiarowski S. Immunological characterization of eristostatin and echistatin binding sites on $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ integrins. Biochem J 1996;317:817–25.
[29] Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labeled Annexin V. J Immunol Methods 1995;184:39–51.
[30] Khosravi E, Elena PP, Hariton C. Allergic conjunctivitis and uveitis models: reappraisal with some marketed drugs. Inflamm Res 1995;44:47–54.
[31] Izushi K, Nakahara H, Tai N, Mio M, Watanabe T, Kamei C. The role of histamine H(1) receptors in late-phase reaction of allergic conjunctivitis. Eur J Pharmacol 2002;440: 79–82.
[32] Strath M, Warren DJ, Sanderson CJ. Detection of eosinophils using an eosinophil peroxidase assay. Its use as an assay for eosinophil differentiation factors. J Immunol Methods 1985;83:209–15.
[33] Pachter JA, Zhang R, Mayer-Ezell R. Scintillation proximity assay to measure binding of soluble fibronectin to antibody-captured alpha 5 beta 1 integrin. Anal Biochem 1995;230:101–7.
[34] Chen LL, Whitty A, Lobb RR, Adams SP, Pepinsky RB. Multiple activation states of integrin alpha4beta1 detected through their different affinities for a small molecule ligand. J Biol Chem 1999;274:13167–75.
[35] Sharif NA, Xu SX, Miller ST, Gamache DA, Yanni JM. Characterization of the ocular antiallergic and antihistaminic effects of Olopatadine (AL-4943A), a novel drug for treating ocular allergic diseases. J Pharmacol Exp Ther 1996;278:1252–61.
[36] Kimani G, Tonnesen MG, Henson PM. Stimulation of eosinophil adherence to human vascular endothelial cells in vitro by platelet-activating factor. J Biol Chem 1988;140:3161–6.
[37] Dobrina A, Menegazzi R, Carlos TM, Nardon E, Cramer R, Zacchi T, et al. Mechanisms of human eosinophil adherence to cultured vascular endothelial cells. J Clin Invest 1991;88:20–6.
[38] Higashimoto I, Chihara J, Kawabata M, Nakajima S, Osame M. Adhesion to fibronectin regulates expression of intercellular adhesion molecule-1 on eosinophilic cells. Int Arch Allergy Immunol 1999;120(Suppl. 1):34–7.
[39] Jiang H, Williams GJ, Dhib-Jalbut S. The effect of interferon-beta 1b on cytokine-induced adhesion molecules expression. Neurochem Int 1997;30:449–53.
[40] Sigal A, Bleijs DA, Grabovsky V, van Vliet SJ, Dwir O, Figdor CG, et al. The LFA-1 integrin supports rolling adhesions on ICAM-1 under physiological shear flow in a permissive cellular environment. J Immunol 2000;165:442–52.
[41] Whitcup SM, Chan CC, Kozhich AT, Magone MT. Blocking ICAM-1 (CD54) and LFA-1 (CD11a) inhibits experimental allergic conjunctivitis. Clin Immunol 1999;93:107–13.

[42] Pesce G, Ciprandi G, Buscaglia S, Fiorino N, Salmaso C, Riccio AM, et al. Preliminary evidence for 'aberrant' expression of the leukocyte integrin LFA-1 (CD11a/CD18) on conjunctival epithelial cells of patients with mite allergy. Int Arch Allergy Immunol 2001;125:160–3.
[43] Fukuishi N, Matsuhisa M, Shimono T, Murata N, Iwanaga M, Sagara H, et al. Inhibitory effect of olopatadine on antigen-induced eosinophil infiltration and the LFA-1 and Mac-1 expression in eosinophils. Jpn J Pharmacol 2002;88:463–6.
[44] Katelaris CH. Ocular allergy: implications for the clinical immunologist. Ann Allergy Asthma Immunol 2003;90:23–7.
[45] Ono SJ, Abelson MB. Allergic conjunctivitis: update on pathophysiology and prospects for future treatment. J Allergy Clin Immunol 2005;115:118–22.
[46] Noble S, McTavish D, Levocabastine. An update of its pharmacology, clinical efficacy and tolerability in the topical treatment of allergic rhinitis and conjunctivitis. Drugs 1995;50:1032–49.
[47] Fujihara T, Takeuchi T, Sato K, Tsubota K. Flow cytometric analysis of surface antigens on human conjunctival epithelial cells. Ophthalmic Res 1997;29:103–9.
[48] Charlesworth EN, Massey WA, Kagey-Sobotka A, Norman PS, Lichtenstein LM. Effect of H1 receptor blockade on the early and late response to cutaneous allergen challenge. J Pharmacol Exp Ther 1992;262:964–70.
[49] Feinberg G, Stokes TC. Application of histamine-induced conjunctivitis to the assessment of a topical antihistamine, levocabastine. Int Arch Allergy Appl Immunol 1987;82: 537–8.
[50] Kamei C, Izushi K, Tasaka K. Inhibitory effect of levocabastine on experimental allergic conjunctivitis in guinea pigs. J Pharmacobiodyn 1991;14:467–73.
[51] Takada Y, Elices MJ, Crouse C, Hemler ME. The primary structure of the alpha 4 subunit of VLA-4: homology to other integrins and a possible cell-cell adhesion function. EMBO J 1989;8:1361–8.