

Short communication

Antiallergic activity of topical lodoxamide on *in vivo* and *in vitro* models

Ciprandi G, Buscaglia S, Catrullo A, Paolieri F, Riccio AM, Fiorino N, Canonica GW. Antiallergic activity of topical lodoxamide on *in vivo* and *in vitro* models.

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Lodoxamide is an antiallergic drug acting as a mast-cell stabilizer, which is effective in the treatment of allergic conjunctivitis. The study aimed to evaluate the effect of lodoxamide eye-drops on the inflammatory early-phase reaction (EPR) changes induced by allergen-specific conjunctival challenge (ASCC). This was a cross-over, double-blind, placebo-controlled, randomized study, including 10 outpatients suffering from allergic rhinoconjunctivitis due to *Parietaria judaica*. Patients received one drop of lodoxamide tromethamine 0.1% or placebo 30 min before each ASCC. Clinical evaluation and cytologic assessment were done at baseline and 30 min after each ASCC. Lodoxamide induced a reduction in total symptom score and hyperemia during the EPR ($P < 0.05$). Lacrimation, itching/burning, and eyelid swelling were only slightly (nonsignificantly) reduced. Lodoxamide induced a reduction in the total number of inflammatory cells and neutrophils during the EPR ($P < 0.02$). Eosinophil and lymphocyte number and ICAM-1 expression showed only a slight, not statistically significant decrease. Placebo did not affect the studied parameters. Lodoxamide reduced early clinical events and cellular changes after ASCC consistently with its activity as mast-cell stabilizer. Moreover, lodoxamide was able to downregulate *in vitro* ICAM-1 expression on the continuously cultured, differentiated conjunctival cell line WK. This was shown both in basal conditions ($P < 0.05$) and upon interferon-gamma stimulation ($P < 0.03$), although at high concentration.

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Mast-cell stabilizers have been widely recommended in the treatment of the allergic diseases (1). Lodoxamide, 2,2'-[(2-chloro-5 cyan-1,3-phenylene) diimino]] bis(2-oxoacetic acid) tromethamine salt, is a mast-cell stabilizer initially proposed for the prophylactic treatment of asthma (2). Several *in vitro* and *in vivo* studies have been performed in animal and human models. Lodoxamide *in vitro* interferes with calcium influx during mast-cell activation, thus inhibiting release of mediators of immediate hypersensitivity (3), and it is a potent inhibitor of rat-lung xanthine oxidase (4).

Lodoxamide inhibits histamine release from rodent mast cells *in vitro* (5). Radioligand binding studies with lodoxamide at 10 mM did not show

any interaction with adenosine A1 and A2 receptors; α - and β -adrenergic receptors; excitatory and inhibitory amino-acid receptors; Ca^{2+} , K^{+} , and Cl^{-} channel proteins; serotonin 1 and 2 receptors; histamine H_1 -receptors; dopamine 1 and 2 receptors; and cholinergic, prostanoid, and opiate receptors (6). The lack of significant interaction with such relevant receptors supports the finding that its activity is associated with mast-cell mediator release inhibition and not end-organ antagonism of released mediators. As far as clinical studies are concerned, lodoxamide is effective in the clinical management of allergic conjunctivitis and reduces inflammatory cell infiltrate in patients with seasonal allergic conjunctivitis (7).

The present study aimed to evaluate lodoxamide antiallergic activity according to two study models. The former study evaluated the possible effect of topical lodoxamide (as single dose as in our protocols) on clinical and inflammatory changes (i.e., inflammatory cell infiltrate and ICAM-1 expression on conjunctival epithelium) after allergen-specific conjunctival challenge (ASCC), a safe and reproducible experimental model of allergic conjunctivitis (7). The latter study evaluated the possible effect of lodoxamide *in vitro* on ICAM-1 expression and release of its soluble form by a continuously cultured, differentiated, conjunctival epithelial cell line, WK.

Material and methods

In vivo study

Study design. This was a cross-over, placebo-controlled, double-blind, randomized study. ASCC was performed as in our previous study (7).

Screening test. A volume of 10 µl of diluted allergen was instilled into the lower conjunctival sac in the right eye. Every 20 min, increasing doses of allergen (10, 50, 100, 150, 300, and 600 AUR/ml) were applied until an early clinical reaction was elicited (a total symptom score >7 was considered positive for early-phase reaction [EPR], as previously described) (7), and, thus, the EPR-provoking dose was defined. One week later, patients, randomly assigned to treatments, alternatively received lodoxamide 0.1% or placebo eye-drops, one drop in the left eye, 30 min before ASCC, performed with the EPR provoking dose. By the same procedure, 1 week later, patients alternatively received lodoxamide or placebo eye-drops, and 30 min later the ASCC was repeated. Clinical conjunctival evaluation and cytologic assessment were performed at baseline and 30 min after ASCC. Conjunctival hyperemia, lacrimation, itching/burning, and swelling of eyelids were scored on an arbitrary four-point rating scale, from 0 to 3 (0 = absent, 1 = mild, 2 = moderate, 3 = severe). A conjunctival scraping for cytologic assessment was obtained from both eyes. The number of inflammatory cells, i.e., neutrophils, eosinophils, and lymphocytes, was counted individually and as a total number of cells in each microscope field; data were expressed as a mean of 10 fields (7). At least 200 cells for each specimen were examined. Slides were examined by two investigators blinded to the identity of the sample. ICAM-1 mAb 84H10 (Immunotech, Marseille, France) was used for immunohistochemical staining of conjunctival epithelial cells. A sensitive immunoenzymatic alkaline

phosphatase-monoclonal antialkaline phosphatase complex procedure, following a procedure modified from Cordell et al. (8), was employed to detect ICAM-1 expression on conjunctival epithelium (7). All possible adverse events spontaneously reported by the patients were carefully noted and evaluated.

Study subjects. Ten *Parietaria judaica*-sensitive subjects (one man and nine women, aged 18–55 years), suffering from seasonal allergic rhinoconjunctivitis, were enrolled and underwent screening ASCC outside the pollen season. All patients had no other ocular diseases, and they did not wear contact lenses; patients with previous documented allergy to the studied drug and women of childbearing potential, or who were lactating were excluded. No topical or systemic drugs were allowed for 1 month before and during the study period. All subjects gave their informed consent, and the trial was approved by our departmental ethics committee.

In vitro study

Cell line. The differentiated, continuously cultured, conjunctival, epithelial cell line WK (Wong-Kilbourne-WK, ATCC CCL 20.2) was maintained in medium 199 (Gibco, Grand Island, NY, USA) supplemented with penicillin 100 U/µl, streptomycin sulfate 100 mg/ml, glutamine 2 mM/l, and 10% heat-inactivated (56°C, 30 min) fetal calf serum (hereafter referred to as culture medium). WK cells were adherent to plastic and were removed from culture flasks by rinsing the monolayers with Ca²⁺- or Mg²⁺-free HBSS (Gibco) and then incubating with trypsin (0.25%) and EDTA (1 mM) for 5 min. The cells were centrifuged and resuspended in culture medium.

Cultures. After resuspension in culture medium, WK cells were cultured in 3 ml of media in six-well tissue-culture dishes (Costar, Italy) at 1×10⁶ cells/well for 24 h in the presence or absence of interferon-gamma (IFN-γ) 500 U/ml (Genzyme, Boston, MA, USA) and of lodoxamide at 0.32, 3.2, and 32 mM/ml (corresponding to 0.01, 0.1, and 1% concentrations, respectively). After 24 h, confluent WK cells were harvested in unicellular suspension with a disposable cell scraper (Costar, Italy) for measurement of ICAM-1 and CD29 (Immunotech, Marseille, France) surface expression by immunofluorescence flow cytometry. The 24-h supernatants were collected, immediately frozen at –70°C, and stored until they were assayed for soluble ICAM-1 content. Four separate experiments were performed.

Flow cytometry analysis. The cells were washed twice by centrifugation (500 g) in PBS, pH 7.2, containing 5% FCS, at 4°C. The cells were then adjusted to 10^6 cells/ml. ICAM-1 or CD29 mAb (primary antibodies, 1 : 100 dilution) were added to a 0.1-ml cell suspension. The cultures were then vortexed and incubated for 30 min at 4°C. The cells were incubated for 30 min at 4°C in culture medium supplemented with developing antibody, FITC-labeled antimouse immunoglobulin antiserum (GAM-FITC, Coulter, Imagine srl, Italy), at a final dilution of 1/100. The cells were washed and resuspended in 500 µl culture medium. The samples were then analyzed by flow cytometry with an Epics Elite cytofluorometer (Coulter, Italy).

Soluble ICAM-1 assay. ELISA of sICAM-1 was performed with a quantitative specific enzyme immunoassay (T Cell Diagnostics, Inc, Cambridge, MA, USA). Twenty-four-hour supernatants and standard dilutions were separately assayed in duplicate. ELISA reaction was read at a wavelength of 490 nm with a microplate autoreader. Results were the means of duplicate samples. The sensitivity of the ICAM-1 ELISA assay was 0.3 ng/ml.

Statistical analysis

Statistical analysis of the *in vitro* and *in vivo* results was done with the Mann-Whitney test for independent data to test differences between groups, as in our previous study (7). A probability of 0.05 or less was considered statistically significant.

Results

In vivo study

The total symptom score and hyperemia were significantly less severe after lodoxamide administration than after placebo one and screening ASCC ($P < 0.05$) (Fig. 1). Cellular infiltrate (total number of inflammatory cells) was significantly reduced after lodoxamide administration compared to placebo one and screening ASCC ($P < 0.02$) (Fig. 2A). In the analysis of single cell type, neutrophils were significantly reduced by lodoxamide compared to placebo and screening ASCC ($P < 0.02$) (Fig. 2B). Lacrimation, itching/burning, and eyelid swelling were slightly reduced, although not statistically significantly, as were eosinophil and lymphocyte number and ICAM-1 expression on conjunctival epithelium. Both lodoxamide and placebo eye-drops were well tolerated, and no adverse events were reported at any time during the study.

In vitro study

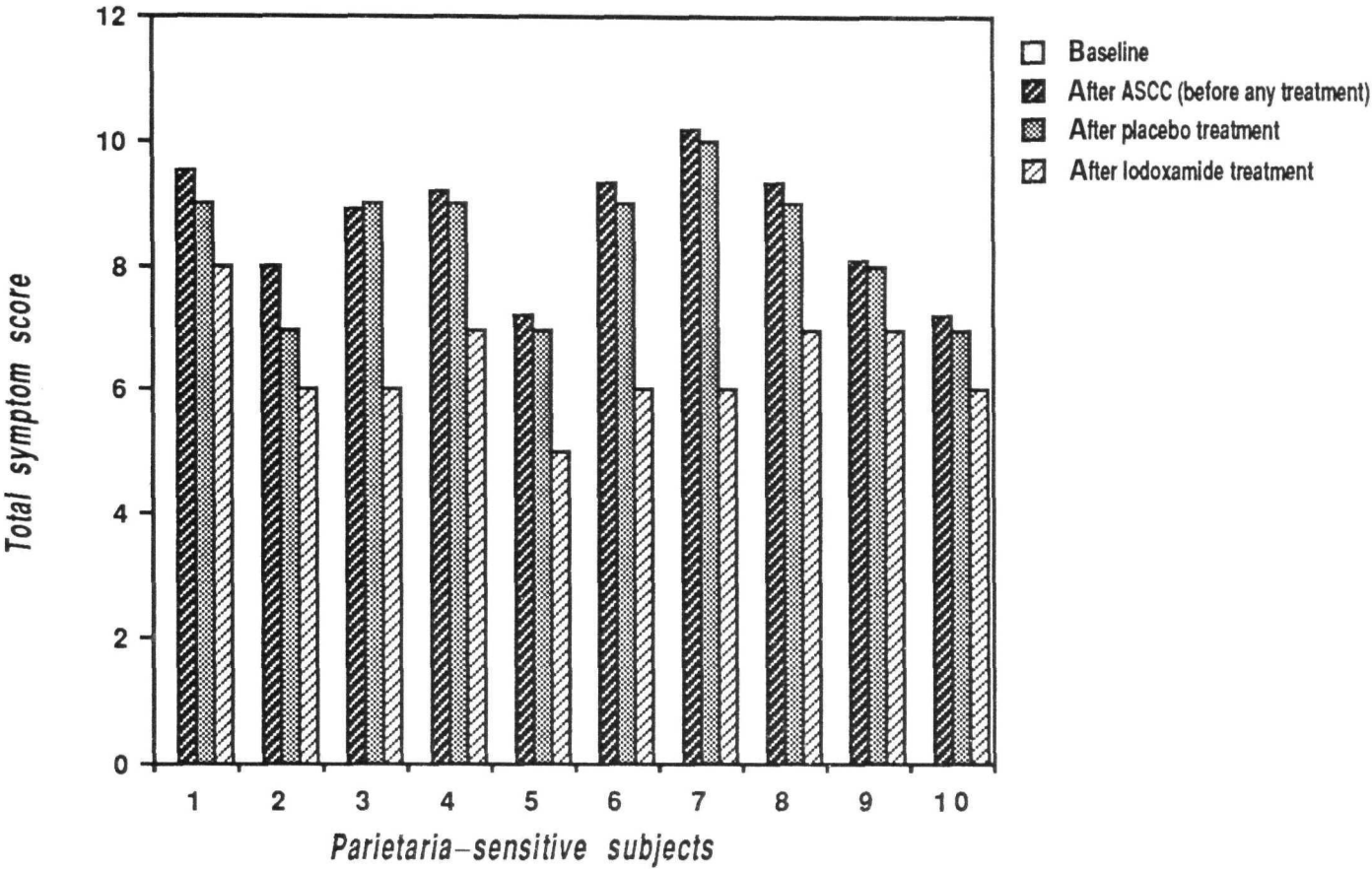
Lodoxamide at the concentrations of 0.32 and 3.2 mM/ml (corresponding to 0.01 and 0.1 concentrations, respectively) did not affect ICAM-1 expression (Fig. 3). On the contrary, lodoxamide at the concentration of 32 mM/ml (corresponding to 1% concentration) did inhibit ICAM-1 expression on WK cells in basal condition ($P < 0.05$) and on IFN- γ -stimulated cells ($P < 0.03$). Lodoxamide at all concentrations did not affect the CD29 surface expression on WK conjunctival epithelial cells (data not shown). Finally, WK cell culture with lodoxamide at all concentrations did not show any significant decrease in level of sICAM-1 release.

Discussion

It is generally accepted that the initial step of allergic reaction requires mast-cell activation and that the vast majority of the typical symptoms of allergic conjunctivitis are mediated through histamine (9). Consequently, it is not surprising that mast-cell stabilizers can find their therapeutic place in the treatment of allergic diseases (1).

Lodoxamide is a mast-cell stabilizer, effective in the prevention of bronchostenotic response after allergen challenge, and also in the treatment of allergic conjunctivitis in acute and long-term therapy (10). It has previously been demonstrated that lodoxamide is effective in reducing clinical and inflammatory parameters in patients with seasonal allergic conjunctivitis (11). The present study aimed to evaluate its antiallergic activity. The results show that a single dose of topical lodoxamide can prevent partially clinical and inflammatory events induced by allergen challenge. Particularly, lodoxamide treatment reduced neutrophil migration into the site of allergic inflammation, which is an early marker of inflammation during allergic reaction (1, 7). Although a large body of data is available on lodoxamide, its mechanism of action needs to be fully investigated. It might be hypothesized that lodoxamide affects only mast-cell activation, probably inducing inhibition of mediator release. It is likely that clinical signs (such as hyperemia) and neutrophil migration were reduced through a reduction of proinflammatory mediator release. As a matter of fact, as already mentioned, lodoxamide does not have antireceptor activity. The slight effect produced on other clinical and cytologic parameters could be related to the shortness of the treatment, just a single dose. Nevertheless, a dose-dependent effect of lodoxamide in preventing clinical and cytologic changes due to ASCC might be hypothesized. On the other hand, *in vitro* data show that lodoxamide, though at high concentration,

A



B

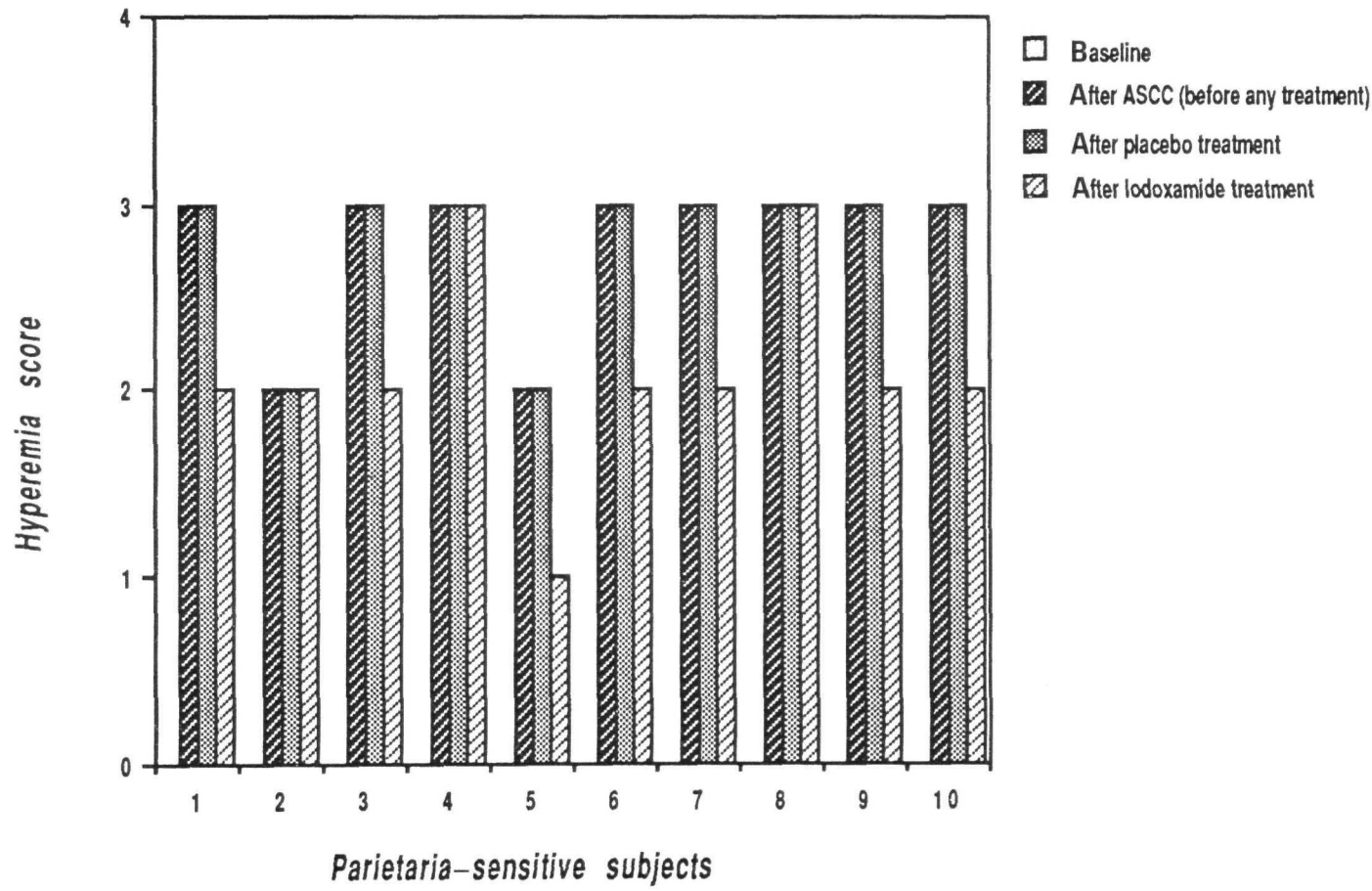
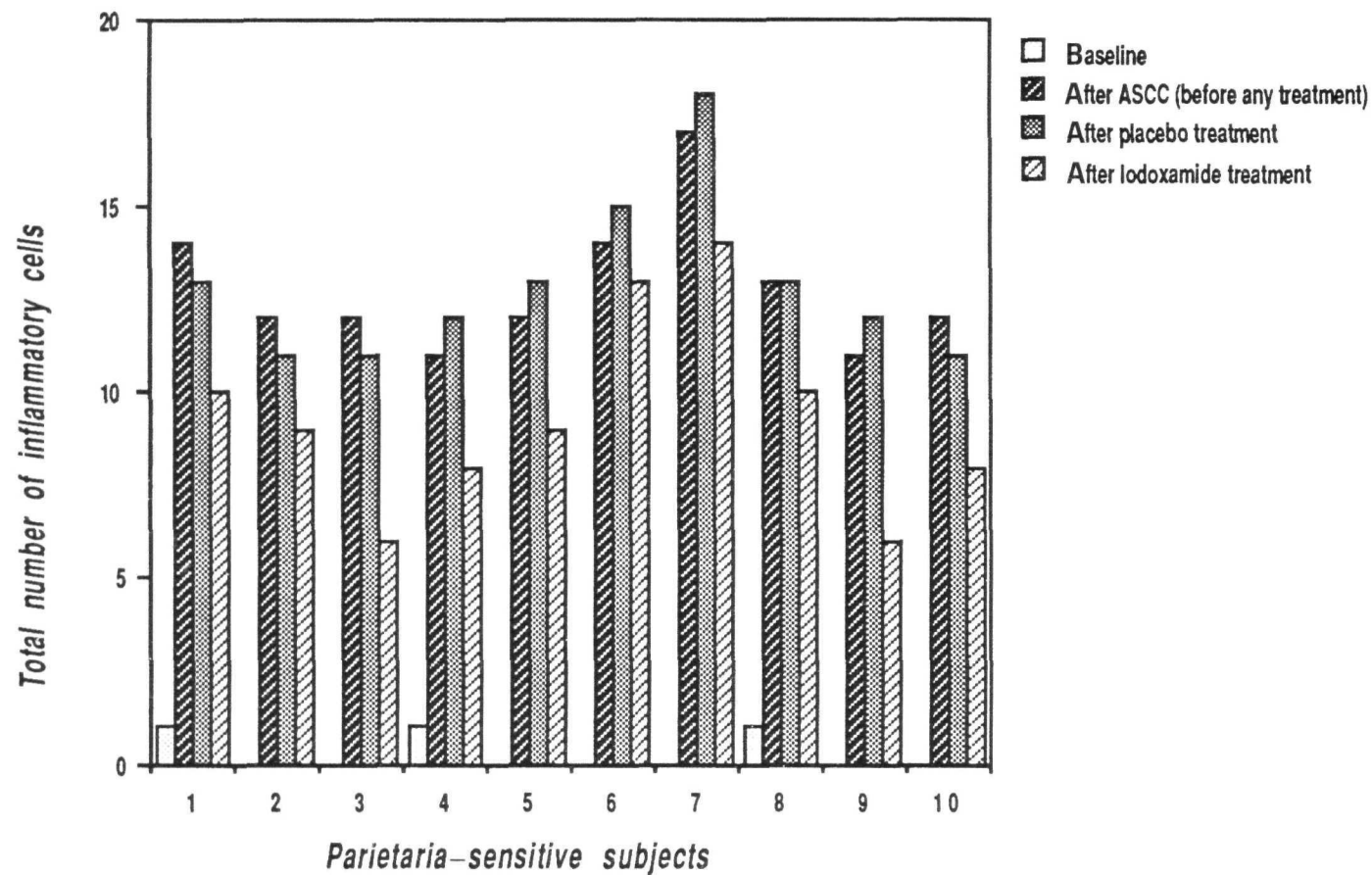


Fig. 1. A) Total symptoms score in basal conditions before and after ASCC, and after lodoxamide and placebo treatment ($P < 0.05$). B) Hyperemia score in basal conditions before and after ASCC, and after lodoxamide and placebo treatment ($P < 0.05$).

A



B

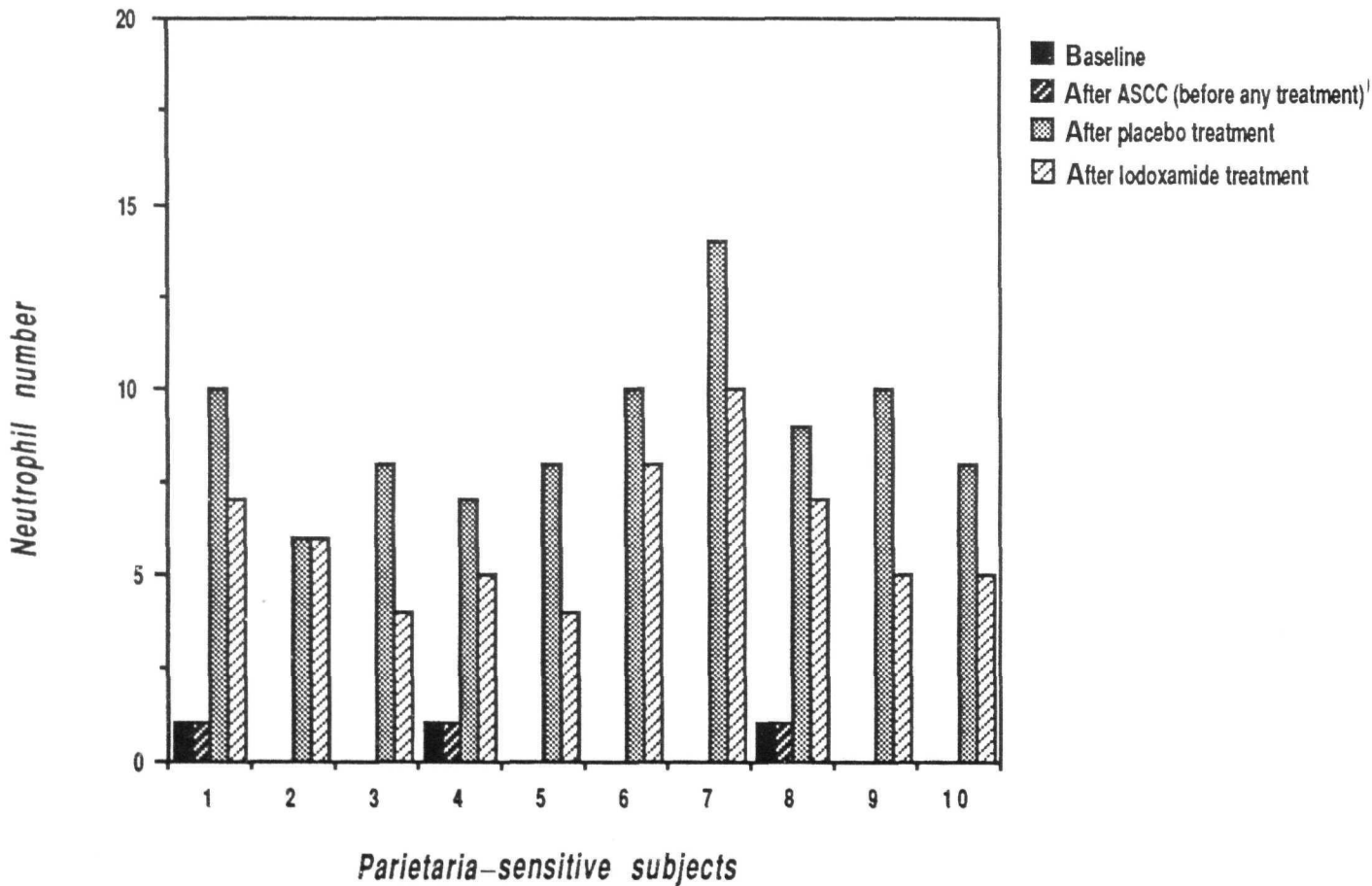
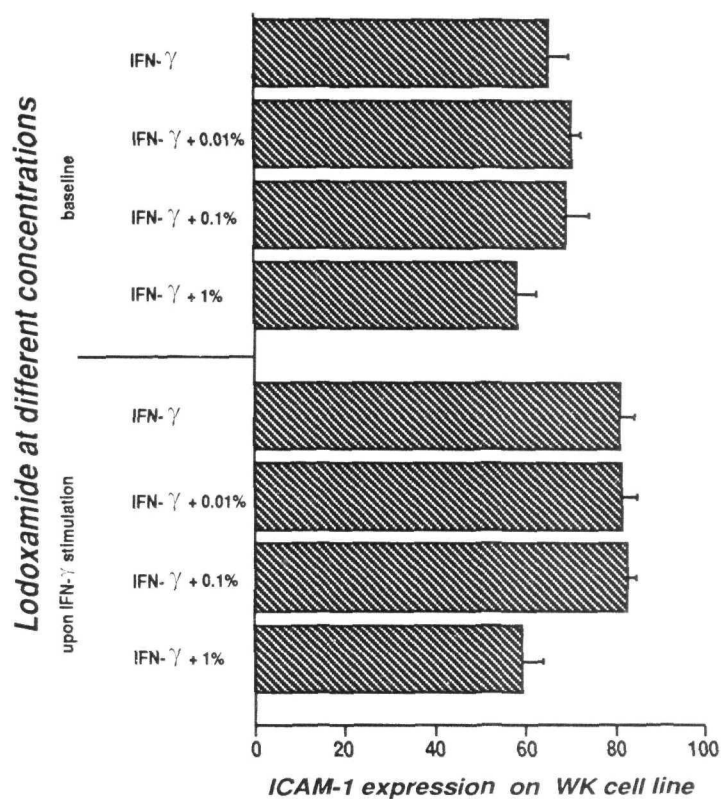


Fig. 2. A) Total inflammatory cell counts in basal conditions before and after ASCC, and after lodoxamide and placebo treatment ($P<0.02$). B) Neutrophil counts in basal conditions before and after ASCC, and after lodoxamide and placebo treatment ($P<0.02$).

A



B

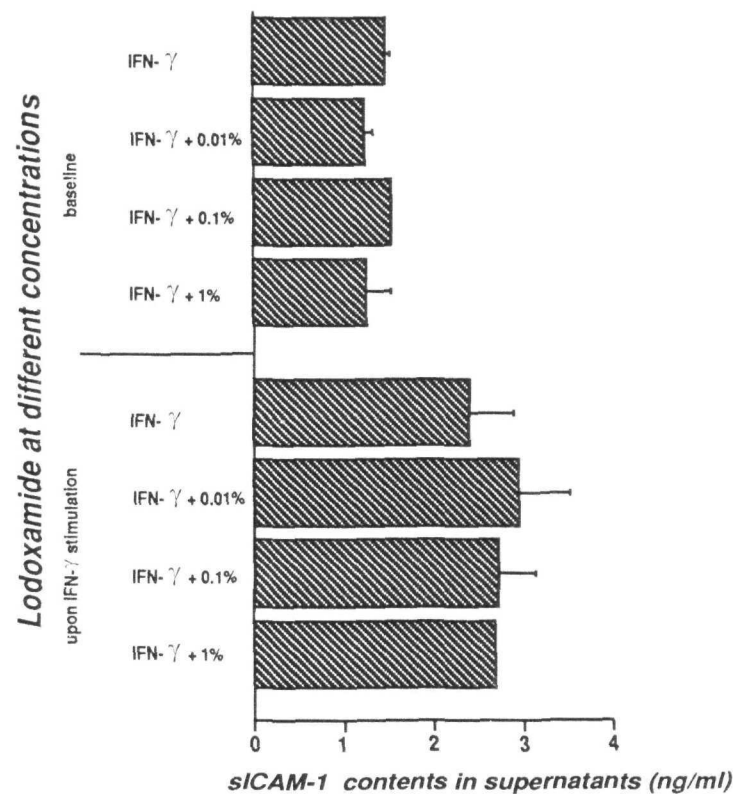


Fig. 3. Surface expression of ICAM-1 on WK cell line after 24-h culture with lodoxamide and without/with 500 U/ml IFN- γ . Composite quantitative fluorescence values, corrected for background, are expressed as mean $\pm$ SEM for four separate experiments. A) Lodoxamide 1% inhibited ICAM-1 expression in basal condition ($P < 0.05$); B) lodoxamide 1% inhibited ICAM-1 expression upon 500 U/ml IFN- γ stimulation ($P < 0.03$).

downregulates ICAM-1 expression on a conjunctival epithelial cell line. All this evidence suggests that future studies should assess the effect on clinical and cytologic parameters of allergic inflammation when long-term treatment is employed, whereas similar compounds, such as nedocromil, *in vitro*, do not affect ICAM-1 expression on conjunctival epithelial cells (12).

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