

Original article

Quantitative flow cytometric analysis of the effects of cetirizine on the expression of ICAM-1/CD54 on primary cultured nasal cells

An *in vitro* flow cytometric model has been developed to evaluate the effects of antiallergic drugs such as cetirizine (CTZ) on the expression of surface molecules on primary cultured normal cells. Quantitative analysis demonstrated that HLA class I and ICAM-1/CD54 molecules are present on both epithelial and stromal cells, and that their expression is strongly enhanced by treatment with interferon-gamma (IFN- γ). Nevertheless, the IFN- γ -mediated upregulation of ICAM-1/CD54 was inhibited by treatment with CTZ, demonstrating a direct effect on both cell types. This finding is particularly interesting because ICAM-1/CD54 is the main rhinovirus receptor, and rhinoviruses are the principal cause of asthma exacerbation in children. Thus, according to data derived from this *in vitro* model, CTZ should have an important role in the reduction of infectious exacerbation of asthma in atopic patients.

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At present there is a general consensus that inflammation is crucial in the pathophysiology of respiratory allergic disease (1). In this context, adhesion molecules, a family of surface structures responsible for cell-to-cell interactions, have recently been described as playing a central role (2). For example, it is well known that ICAM-1/CD54 is deeply involved in virtually all immune reactions in both physiologic and pathologic conditions (3–6). In allergic patients, several studies have demonstrated the strict correlation between ICAM-1/CD54 expression on epithelial cells at the nasal and conjunctival levels and both the severity of clinical manifestations and the amount of allergic pressure (7, 8). In addition, *in vitro* studies have demonstrated that ICAM-1/CD54 is the main receptor for human rhinoviruses (9, 10). This characteristic is of great basic and clinical interest because it has been exhaustively shown that rhinoviruses represent the most frequent agent of the common cold (approximately 50% of cases), and in childhood, the exacerbation of asthma is usually associated with upper respiratory viral infections (11). Cetirizine (CTZ), the carboxylated metabolite of hydroxyzine, is a second-generation H₁-

antagonist with antiallergic properties, as demonstrated by its efficacy in reducing both the accumulation of eosinophils and the expression *in vivo* of ICAM-1/CD54 at the nasal and conjunctival levels after allergenic challenge (12). In addition, we have already demonstrated *in vivo*, by immunocytochemical means, that CTZ is able to downregulate ICAM-1/CD54 on epithelial cells, but it is still unknown whether this is due to direct action on epithelial cells, or whether it occurs through different target cells (12, 13).

Previous studies have demonstrated the possibility of modulating ICAM-1/CD54 on monoclonal cell lines by antiallergic drugs by semiquantitative immunochemistry or immunoenzymatic methods (5). In this study, we established an *in vitro* model consisting of primary cultures of epithelial and stromal cells from surgical specimens of patients who underwent nasal surgery for polyposis and turbinate hypertrophy. The effects of CTZ on the surface expression modulation of ICAM-1/CD54 and HLA I class antigens were assessed by quantitative flow cytometric analysis both in basal conditions and during activation of the tested cells due to the addition of IFN- γ to the medium culture.

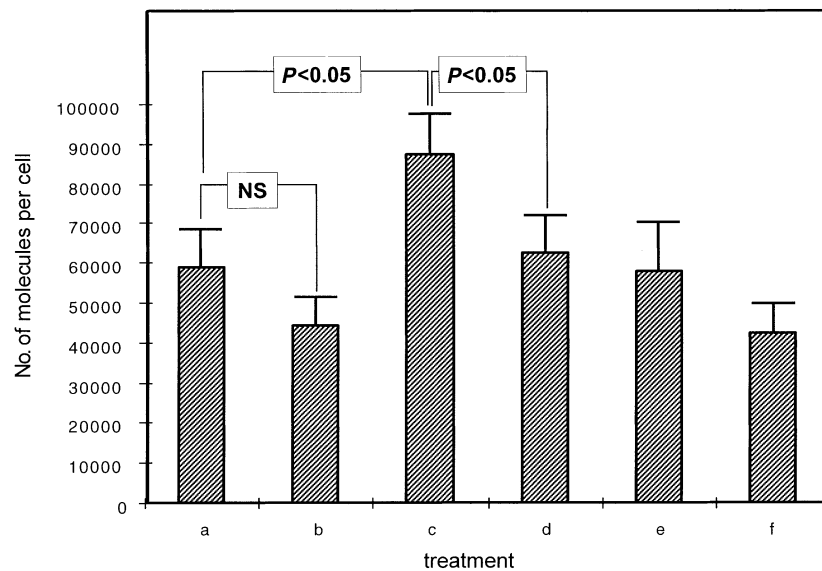


Figure 1. Expression of ICAM-1/CD54 on epithelial cells. Different treatments reported along horizontal axis: a) no treatment; b) 0.1 mg/ml CTZ; c) 500 U/ml IFN- γ ; d) 500 U/ml IFN- γ +0.01 mg/ml CTZ; e) 500 U/ml IFN- γ +0.05 mg/ml CTZ; f) 500 U/ml IFN- γ +0.1 mg/ml CTZ. Vertical axis: no. of molecules/cell $\pm$ SEM. *P*: statistical significance; NS: nonsignificant.

Material and methods

Patients

Six patients (three women and three men, median age 55, range 23–63 years) undergoing nasal surgery for polypoid or turbinate hypertrophy were included in the study. A fragment of nasal mucosa was obtained from each patient. None of the patients had taken any drug (topical or systemic corticosteroids, anti-H₁ receptor antagonists) for at least 2 months. The study was approved by the ethics committee of the University of Genoa (Italy), and all patients signed informed consent forms.

Specimen collection and cell culture

In order to analyze the effect of CTZ on a cellular substrate highly comparable to *in vivo* human tissues, nasal biopsies were placed in sterile conditions in RPMI 1640 medium (HyClone, Cramlington, UK) supplemented with antibiotics (penicillin and streptomycin) and immediately processed in the laboratory. The tissue was mechanically minced into small pieces and then enzymatically dissociated in the presence of collagenase (500 U/ml, Sigma), hyaluronidase (30 U/ml, Sigma), and DNase (10 U/ml, Sigma) for 2 h at 37°C. Single cell suspensions were washed twice in fresh medium and seeded in two different 75-cm² plastic flasks. In order to

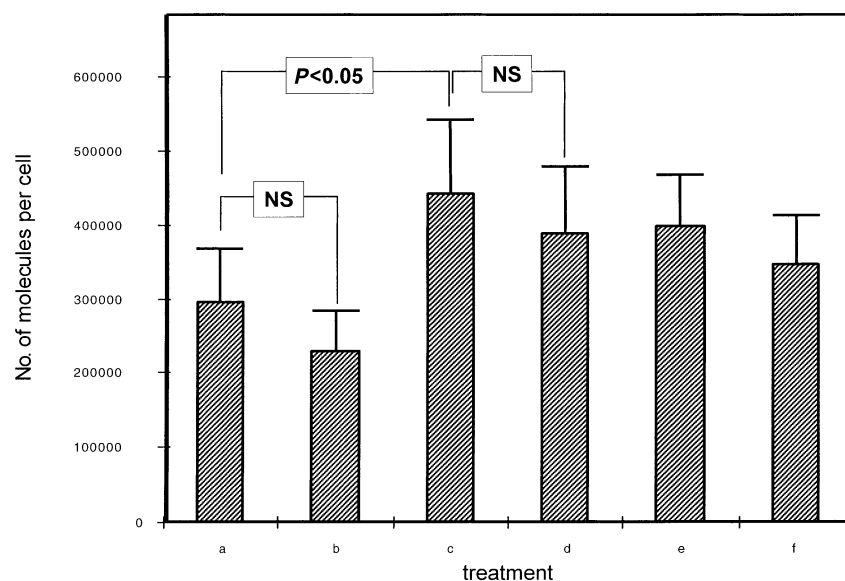


Figure 2. Expression of HLA class I molecules on epithelial cells. See also legend to Fig. 1.

Table 1. Characteristics of epithelial and stromal cells derived from nasal mucosa

Patient no.	Morph	CAM	HLA			ICAM-1/ CD54			HLA			CD54	
			HLA	IFN- γ	CD54	IFN- γ	Morph	CAM	HLA	IFN- γ	CD54	IFN- γ	CD54
1	Ep.	100	470	820	46	61	Fib.	0	456	863	112	157	
2	Ep.	97	537	600	39	93	Fib.	0	207	393	46	77	
3	Ep.	93	140	180	86	127	Fib.	0	520	700	36	95	
4	Ep.	97	350	480	70	82	Fib.	0	393	780	73	88	
5	Ep.	94	176	366	83	96	Fib.	0	152	211	18	64	
6	Ep.	95	90	203	30	65	NE	—	—	—	—	—	

Morph: morphology; CAM: % of CAM 5.2 (cytokeratin)-positive cells; Ep: epithelial; Fib: fibroblast; HLA: no. of HLA class I molecules $\times 1000$ on cell surface; CD54: no. of CD54 molecules $\times 1000$ on cell surface; NE: not expanded.

obtain the expansion of either the stromal or epithelial components, one flask was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), glutamine, and antibiotics, while the other flask was cultured with a mixture of DMEM-HAM F-12 medium supplemented with 10% FBS, glutamine, antibiotics, and growth factor to support the epithelial component, as previously described (14). After 1 week, culture growth was checked and the cells were used immediately. The presence of epithelial cells was detected by intracytoplasmic immunofluorescence assay with an anticytokeratin monoclonal antibody (CAM 5.2, Becton-Dickinson, San Jose, CA, USA) and flow cytometry.

Stimulation of cultured cells by IFN- γ and inhibition by CTZ

To analyze the effect of CTZ on both epithelial and stromal cells derived from human nasal mucosa, primary cultured cells were trypsinized, resuspended in fresh culture medium, and counted by the trypan blue dye exclusion test. A total of 5×10^5 cells were seeded onto six-well plates in order to obtain an exponentially growing culture expressing a high number of surface molecules (14). Three different doses of CTZ (0.01, 0.05, and 0.1 mg/ml) were added to plated cells and incubated for 24 h at 37°C. In addition, in order to simulate the events during allergic inflammation or viral infection, the expression of surface molecules on target cells was upregulated with 500 UI/ml of IFN- γ (14).

Controls consisted of wells treated only with IFN- γ , wells treated only with the highest dose of CTZ (0.1 mg/ml), and wells without drugs. All the experiments were performed in triplicate.

DNA analysis

To evaluate the potential proliferative damage induced by CTZ on primary cultures of nasal mucosa cells, propidium-iodide staining and flow cytometry were employed, as previously described (14). Different cell-cycle phases were calculated with Multicycle software on DNA histograms derived from an Epics XL flow cytometer.

Phenotypic analysis of cells

Immunofluorescence and flow cytometry were employed to calculate the number of surface molecules on cultured cells. The following fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies (mAbs) were used: W6/32, which recognizes a monomorphic determinant of the HLA class I molecule (15), and anti-CD54 (Immunotech, Luminy, France) directed against the ICAM-1/CD54 adhesion molecule. Briefly, 0.05 ml containing 1×10^5 cells was incubated with 10 ng/ml mAbs for 30 min at 4°C, followed by extensive washing and analysis on a Coulter EPICS XL flow cytometer. Analyses were carried out on cells gated to exclude nonviable cells.

Surface molecule expression was quantitatively analyzed as previously described (14). Briefly, the number of molecules expressed on the cell surface was calculated with different standard beads with a known number of FITC equivalent ones (Coulter Scientific, Miami, FL, USA) on the basis of the molar protein/FITC ratio, calculated as previously described (16).

Statistical analysis

Statistical analysis for intergroup comparisons was performed by the nonparametric Mann-Whitney U-test. *P* values less than 0.05 were considered statistically significant.

Table 2.

Cells	Molecule	NT	CTZ	IFN- γ 500 U/ml	IFN- γ 500 U/ml + CTZ	IFN- γ 500 U/ml + CTZ	IFN- γ 500 U/ml + CTZ
			0.1 mg/ml		0.01 mg/ml	0.05 mg/ml	0.1 mg/ml
Epithelial	ICAM/CD54	5.9 ± 0.97	4.4 ± 0.77	8.7 ± 0.98	6.23 ± 0.96	6.01 ± 1.13	42 ± 7.2
	HLA I	29.3 ± 7.5	22.7 ± 5.6	44.2 ± 10	38.8 ± 9.2	39.6 ± 7.2	34.5 ± 6.9
Stromal	ICAM/CD54	5.7 ± 1.63	4.4 ± 0.73	9.62 ± 0.16	6.99 ± 1.04	6.08 ± 0.76	54.1 ± 7.9
	HLA I	34.5 ± 7.1	35.54 ± 5.4	58.9 ± 12.3	56.2 ± 12	43.5 ± 8.1	46.9 ± 11.5

Data are expressed as number of molecules $\times 10^4$ per cell (mean $\pm$ SEM).

Table 3. DNA quantitative analysis

Treatment	G0/G1	S	G2/M
A	76.5 (69–82)	6.5 (5–18)	10.5 (9–12)
B	74 (69–78)	9 (6–17)	11.5 (9–14)
C	76.5 (69–77)	8 (4–17)	11.5 (8–14)
D	77 (70–81)	8 (4–15)	12.5 (7–13)
E	74.5 (71–81)	8.5 (4–16)	11.5 (8–14)
F	77.5 (69–85)	5.5 (2–17)	9.5 (7–13)
Statistics	NS	NS	NS

A) No treatment; B) treatment with 500 ng/ml of IFN- γ ; C) treatment with 500 ng/ml of IFN- γ and CTZ 0.01 mg/ml; D) treatment with 500 ng/ml of IFN- γ and CTZ 0.05 mg/ml; E) treatment with 500 ng/ml of IFN- γ and CTZ 0.1 mg/ml; F) treatment with CTZ 0.1 mg/ml. G0/G1: median and range of percentages of cells in G0/G1 phase; S: median and range of percentages of cells in S phase; G2/M: median and range of percentages of cells in G2/M phase; NS: nonsignificant.

Results

Cultures were started from tissue specimens derived from six different patients. Each sample was processed in order to obtain the expansion of both epithelial and stromal components. Therefore, the effect of CTZ was investigated separately on the two different cellular populations.

Epithelial cells were obtained in all samples, whereas stromal cells were obtained in only five out of six samples (one was excluded because of bacterial contamination). Morphologic aspects as well as reaction to anticytokeratin mAb clearly demonstrated the different origins of the two groups of cell cultures (Table 1).

The quantitative analysis of surface molecules on cultured cells showed a relatively constant expression of HLA class I in virtually all resting cultures. On the contrary, treatment with IFN- γ upregulated antigen expression in both epithelial and stromal cells (Table 1). This effect confirmed the susceptibility of cultured cells

to respond to stimuli that usually increase the expression of the relevant surface molecules, and therefore confirmed that the functional capabilities of cultured epithelial and stromal cells were intact. As far as surface molecules are concerned, no significant differences were observed between untreated and CTZ-alone-treated cells, although a slight reduction of the expression of ICAM-1/CD54 surface molecules was detected. In the same experiments, the lowest doses of CTZ were able to reduce significantly the ICAM-1/CD54 overexpression induced by the treatment with IFN- γ on both epithelial (mean molecules per cell: from $87 \times 10^3 \pm 8.9 \times 10^3$ SEM to $62.3 \times 10^3 \pm 9.6 \times 10^3$ SEM; $P=0.03$) and stromal (mean molecules per cell: $96.2 \times 10^3 \pm 16.6 \times 10^3$ SEM to $69.9 \times 10^3 \pm 10.4 \times 10^3$ SEM; $P<0.05$) cells (Table 2; Figs. 1 and 3). The more concentrated doses of CTZ tested further reduced ICAM-1/CD54 IFN- γ -induced upregulation on both cell lines, demonstrating a dose-dependent effect. On the contrary, no significant effects of CTZ were observed in the modulation of HLA class I antigens (Table 2; Figs. 2 and 4).

The quantitative analysis of DNA by propidium-iodide staining on the cells from the different treatments showed no differences in the percentages of cellular cycle phases. Thus, CTZ did not affect the percentages of the cells in the different phases of the cycle (Table 3). This observation suggests that the surface molecule modulation observed was not related to the proliferative features of the cells.

Discussion

We describe the development of an *in vitro* model suitable for the quantitative analysis of the modification of surface molecules on low-passage cultured normal

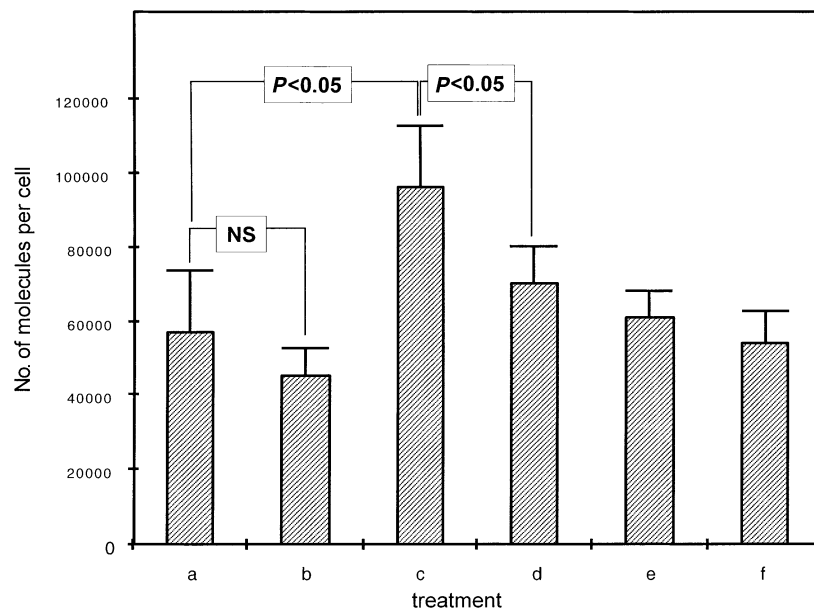


Figure 3. Expression of ICAM-1/CD54 class molecules on stromal cells. See also legend to Fig. 1.

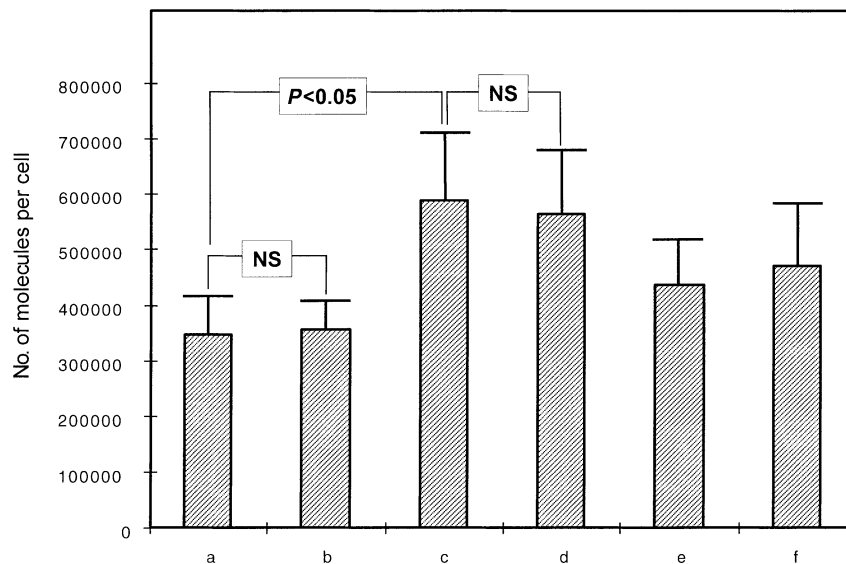


Figure 4. Expression of HLA class I molecules on stromal cells. See also legend to Fig. 1.

cells. The advantages of this approach are evident. First, cells isolated from normal mucosae better represent the *in vivo* conditions than do established cancer cell lines. Second, the analysis of the effects on both epithelial cells and stromal cells (fibroblasts) allows further analysis of the effects observed on two different components of respiratory mucosa. Third, the direct effect of the drug on the tested cells in terms of the expression of surface molecules is analyzed; thus, the presence of a dose-dependent mechanism can be evaluated. Fourth, quantitative evaluation based on the calculation of the number of mean molecules per cell, instead of the mean fluorescence values, allows more accurate measurement of the modulation, and comparative studies with different modulators can be conducted.

Thus, we evaluated the suitability of our model by analyzing the effect of CTZ on a subset of surface molecules. CTZ presented a weak effect in reducing ICAM-1/CD54 expression from basal conditions; i.e., in non-IFN- γ -stimulated epithelial and stromal cells. This effect was, however, not statistically significant. On the contrary, CTZ was able to reduce significantly the ICAM-1/CD54 overexpression induced by treatment with proinflammatory cytokines (such as IFN- γ). IFN- γ is produced by lymphoid compartment cells upon exposure to antigens (such as viruses or allergens). Even if IFN- γ were not the only cytokine produced under these conditions, it is still highly representative of the lymphokine cascade that occurs at the immunoreaction site. Thus, the addition of exogenous IFN- γ can accurately mimic the inflammation that occurs after exposure to allergens and infectious agents. Unlike the effects observed on ICAM-1/CD54 expression, no effect of CTZ was observed in modifying the IFN- γ -induced overexpression of HLA I class antigens. Thus, the effect

of CTZ on ICAM-1/CD54 should have some specificity. This observation also suggests that the modulatory effect of CTZ operates only “as needed”, i.e., when inflammatory events are present, and only weakly under basal conditions. We previously demonstrated that CTZ does not affect ICAM-1/CD54 and CD25 expression on lymphocytes upon mitogen stimulation (18), thus confirming that CTZ has no immunosuppressive effect. The downregulation of the induced overexpression of ICAM-1/CD54 on the surface of the epithelial and stromal cells leads to a decreased possibility of interactions between the immunocompetent cells and the tissue components, thus providing a protective effect to cells susceptible to inflammatory cell adhesion. Our demonstration that CTZ may act on fibroblasts, recently described as potential protagonists in the allergic inflammatory process (19), is extremely intriguing. Downregulation at the airway epithelial level should also reduce susceptibility to rhinovirus infections and, in this way, may lead to a reduced frequency of clinical exacerbations, such as asthma attacks.

The effects of CTZ were observed within a range of concentrations of 0.01–0.1 mg/ml, concentrations 10-fold higher than those reached at the plasma peak *in vivo*. This finding did not affect the validity of the model proposed because, in inflamed tissue, the concentration of drug is frequently higher than that at the plasma peak (a finding based upon accumulation and specific organ half-life). In addition, other pharmacologic evidence has been collected *in vitro*, with concentrations unrelated to plasma peak levels (13).

In conclusion, the model proposed is highly suitable for *in vitro* studies on the pharmacologic effects of antiallergic drugs on the expression of surface molecules highly involved in the cell-to-cell relationships. The

quantitative method employed could be used for other molecules, and in comparative studies with different compounds, providing results that would potentially be transferable to *in vivo* conditions. In addition, this method demonstrates a novel activity of CTZ in the modulation of ICAM-1/CD54 overexpression in normal epithelial and stromal cells activated by proinflammatory molecules, such as IFN- γ . The finding that the tissue components investigated are susceptible

to the effects of CTZ suggests that the drug has a targeted organ tropism, at least for the functional features analyzed.

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