

Effect of terfenadine on TNF α release from peripheral blood mononuclear cells during cow's milk allergy

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Summary

Background In infants with cow's milk allergy and intestinal symptoms, peripheral blood mononuclear cells stimulated *in vitro* with cow's milk proteins, secrete large amounts of the proinflammatory cytokine TNF α thus altering intestinal barrier capacity. Terfenadine, an antihistaminic drug, inhibits the release of several inflammatory mediators, including histamine, prostaglandins and leukotrienes.

Objectives To test the potential ability of terfenadine to inhibit TNF α secretion by mononuclear cells from infants with cow's milk allergy.

Methods Mononuclear cells from infants allergic to cow's milk proteins were stimulated *in vitro* for 6 days by a mixture of milk proteins (β -lactoglobulin, α -lactalbumin and casein) with or without terfenadine (0.1–1 μ M) and culture supernatants were assayed for TNF α by enzyme immunoassay. The effect of culture supernatants on intestinal barrier capacity was evaluated by measuring the electrical resistance (index of integrity) of filter-grown HT29–19 A intestinal cells in Ussing chambers.

Results During active cow's milk allergy, mononuclear cells stimulated with cow's milk proteins secreted large amounts of TNF α which significantly reduced the electrical resistance of HT29–19 A intestinal cells. There was a dose-dependent decrease in TNF α secretion in the presence of terfenadine, with a maximal inhibition of 62% of this secretion at 1 μ M. Accordingly, terfenadine-treated mononuclear cells supernatants did not alter the electrical resistance of intestinal HT29.19 A cells.

Conclusion These results indicate that in infants with intestinal dysfunction due to cow's milk allergy, terfenadine is a potent inhibitor of the TNF α secretion induced by sensitizing milk protein antigens. This inhibition prevents the degradation of intestinal function as measured in an intestinal cell line, *in vitro*.

Keywords: terfenadine, mononuclear cells, cow's milk allergy, TNF α , intestinal barrier

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Introduction

Terfenadine is a non-sedating antihistamine frequently used in the treatment of skin and airway allergies. Although it was initially described as a H₁-receptor antagonist, it also displays

mast cell stabilizing properties [1] and anti-inflammatory properties [2,3]. In humans, terfenadine inhibits the release of various mediators *in vivo* [4–6] and *in vitro* [2,7–9]. Indeed, it reduces the secretion and the inflammatory reactions induced by histamine [2,5–8] and arachidonic acid metabolites including prostaglandins D₂ or LTC₄/D₄/E₄ [2,6–10]. In addition, terfenadine down-regulates the expression of adhesion molecules such as ICAM-1 on nasal epithelial cells [11], thus decreasing the recruitment of inflammatory cells. Terfenadine also inhibits the release of neuropeptides

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such as substance P and vasoactive intestinal polypeptide in nasal mucosa [4].

The main target cells for terfenadine are mast cells [2,7], peripheral blood basophils [2,8,10] and eosinophils [8,10].

In the present report, we describe the effect of terfenadine on cytokine release by mononuclear cells (lymphocytes/macrophages) in children allergic to cow's milk. Cow's milk allergy (CMA) is a complaint associated with adverse reactions affecting the skin, airways, and intestine. Clinical reactions are either IgE-dependent, mainly manifested by either immediate symptoms or delayed T cell-mediated reactions. In CMA with gastrointestinal symptoms, in which infants mostly exhibit late reactions and low levels of specific cow's milk protein IgE antibodies, we recently described the role of mononuclear cells [12]. In this investigation, isolated peripheral blood mononuclear cells (PBMC) from infants with CMA and intestinal symptoms, were found to secrete large amounts of TNF α and IL6 in the presence of cow's milk proteins without modifying IL-4 and IFN γ production. Using neutralizing antibodies to these cytokines and h-TNF α , we have shown that TNF α is responsible for the harmful effect on the intestinal barrier capacity measured *in vitro*, using the human intestinal cell line HT29-19 A [12]. The aim of the present study was to investigate the possibility that terfenadine inhibits TNF α release during cow's milk allergy, and the effect of any such inhibition on intestinal barrier capacity.

Materials and methods

Patients

Twenty-three infants were included in this study. Their mean age was 18.9 ± 4.3 months. All were initially diagnosed as being allergic to cow's milk proteins (CMP) as defined by the criteria of the European Society for Gastroenterology and Nutrition [13]. CMA was diagnosed by initial cow's milk protein elimination followed by a positive challenge with an unprocessed cow's milk infant formula. The infants were then put on a cow's milk free diet for at least 3 months, after which they underwent a late rechallenge with the same infant formula. A blood sample of 2–3 mL was collected just before this rechallenge, kept at room temperature and tested within 1 h of collection. Informed consent was obtained from all parents or guardians. The infants were subsequently divided into two groups, according to their clinical reaction to the cow's milk rechallenge. The first group, the 'active CMA group' (mean age 27.4 ± 9.0 months) comprised 10 infants (three of them with high CMP-specific IgE antibodies) who displayed a positive clinical reaction with digestive symptoms (vomiting or diarrhoea). The second group, 'the cured CMA group' (mean age 12.5 ± 2.1 months), comprised 13 infants

who did not react during the late cow's milk rechallenge but had exhibited clinical reactions during at least one previous milk challenge.

PBMC were cultured in a blinded manner with respect to clinical outcome and the children were classified into the active CMA group or cured CMA group according to the clinical outcome.

Isolation and stimulation of Peripheral Blood Mononuclear Cells (PBMC)

PBMC were isolated from EDTA-blood samples using a ficoll gradient (MSL; Eurobio, Les Ulis, France), washed three times with buffer saline, resuspended in the RPMI 1640 culture medium (Eurobio) containing 10% heat-inactivated human blood group AB serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 4 mM glutamine. Cell content was adjusted to 10^6 /mL. Cells were stimulated by a mixture of cow's milk proteins (CMP) comprising β -lactoglobulin, α -lactalbumin and casein at a final concentration of 200 μ g/mL for each, with various amounts of terfenadine (0.1 μ M, 1 μ M) or without terfenadine. Parallel control cultures were run without stimulation or with stimulation by the non-milk protein ovalbumin (Ova) at a final concentration of 200 μ g/mL. Cells were incubated for 6 days in a humidified atmosphere of 5% CO₂. Culture supernatants from unstimulated PBMC or PBMC stimulated by CMP or by CMP plus terfenadine -, respectively, referred to below as Control, CMP and CMP+Terf supernatants—were collected and stored at -80°C until use. Terfenadine was obtained as a dry powder from Marion Merrell Dow France and solubilized in ethanol.

Effect of terfenadine on PBMC viability

In order to detect a possible toxic effect of terfenadine on peripheral blood mononuclear cells, we conducted a numeration of the cells before and after a 6 day-treatment with increasing amounts of terfenadine (0.1, 1 and 10 μ M). The effect of terfenadine vehicle, ethanol, was also tested. We also measured lactic dehydrogenase (LDH) release from PBMC after treatment with terfenadine, as an index of cell membrane suffering.

Briefly, 2×10^6 cell/mL were incubated in the different experimental conditions during 6 days. Cells were centrifuged and LDH was assayed in the culture supernatants. In order to measure the release of intracellular LDH, cells were resuspended in 1 mL RPMI and lysed by triton X100 0.5% during 30 min at 37°C . Terfenadine-induced LDH release as well as total LDH are reported in IU/mL. In another set of experiments, cells were counted before and after a 6 day incubation period in the presence of ethanol or terfenadine alone.

Assay of TNF α in PBMC culture supernatants

Supernatants were assayed for TNF α using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Tebu, Le Perray en Yvelines, France). The limit of sensitivity was 50 pg/mL.

The intestinal cell line HT29–19 A as a model of human intestinal epithelium The human colon carcinoma cell line HT29–19 A, cloned from the HT29 cell line by Augeron and Laboisse [14] and cultured on permeable supports, was used as a model to assess intestinal barrier capacity, as previously described [15]. Between passages 9 and 35, cells were cultured at 37°C in Dulbecco's modified Eagle medium (DMEM, Eurobio, Les Ulis, France) containing 10% heat-inactivated fetal calf serum, 4 mM glutamine and 50 μ g/mL gentamycin, in a humidified atmosphere of 5% CO₂/95% air. The medium was changed daily and cells were seeded at a density of 0.8 10^6 cells/cm² on polycarbonate transwell® filters with a pore diameter of 0.4 μ m and a surface area of 1.13 cm² (Costar, Brumath, France). Cells formed a monolayer that reached a stable electrical resistance of about 150 Ω /cm² after 3 weeks, and were used as from that time.

Measurement of intestinal barrier capacity after treatment with PBMC supernatants

PBMC culture supernatants were applied for 48 h to the basal compartment of transwell-grown HT29–19 A intestinal cells at a final concentration of 10% (v/v). Controls were run to test the direct effect of 0.1 or 1 μ M terfenadine alone and the terfenadine vehicle, ethanol, on intestinal barrier capacity. The inserts were then removed from the culture plates, filters were gently cut out and mounted in Ussing chambers with an exposed area of 0.15 cm². Cell monolayers were exposed on each side to 1.5 mL of oxygenated, thermostated Ringer solution, as previously described [15]. After appropriate correction for fluid and system resistance, monolayers were short-circuited using an automatic voltage-clamp (WPI, Aston, UK), the potential difference was checked at 10 min intervals over 30 min, and electrical resistance (R) was calculated by clamping the monolayer at an imposed potential of 5 mV and applying Ohm's law according to the current deflection. This electrical parameter (R) was considered as an index of epithelial integrity.

Statistical analysis

Data were analysed using the SAS (SAS institute, Cary, NC, USA) package [16]. One-way variance analysis was performed using the general linear model procedure, to compare means and ranges. Non-parametric test (Kruskal–Wallis) was used for small size samples (PBMC viability). Results are expressed as means $\pm$ SE.

Results

Effect of terfenadine on PBMC viability

Figure 1 (a) shows LDH release by PBMC after different treatments. As ethanol is used as the vehicle of terfenadine, its possible toxic effect is tested, as well as that of increasing amounts of terfenadine. As expected, total LDH content was the same in all experimental conditions but a significant

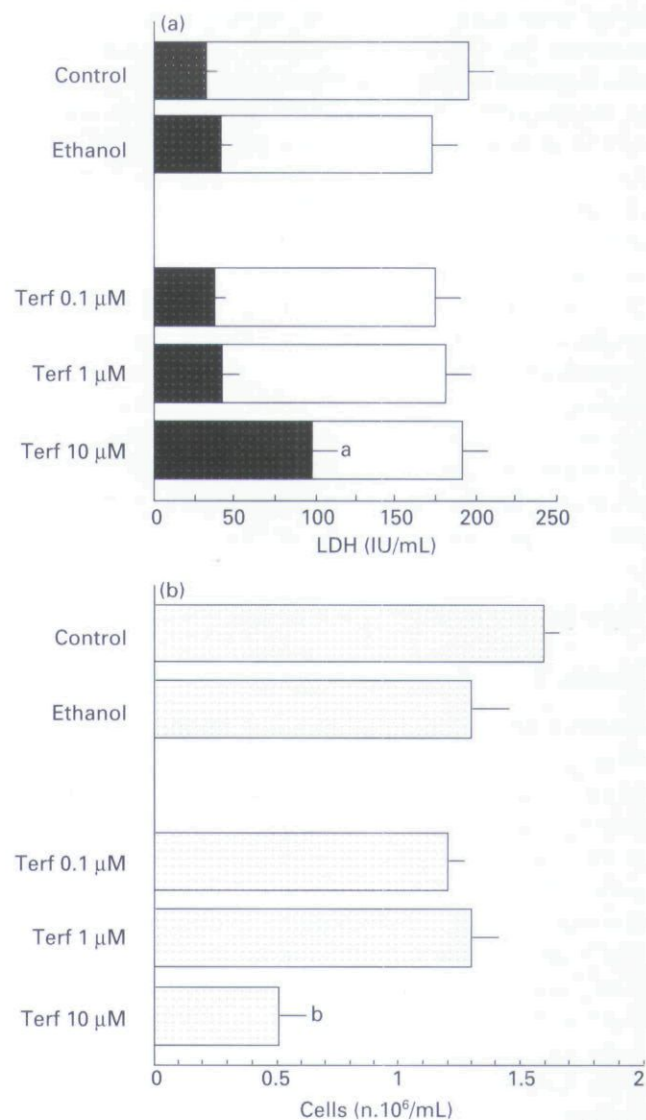
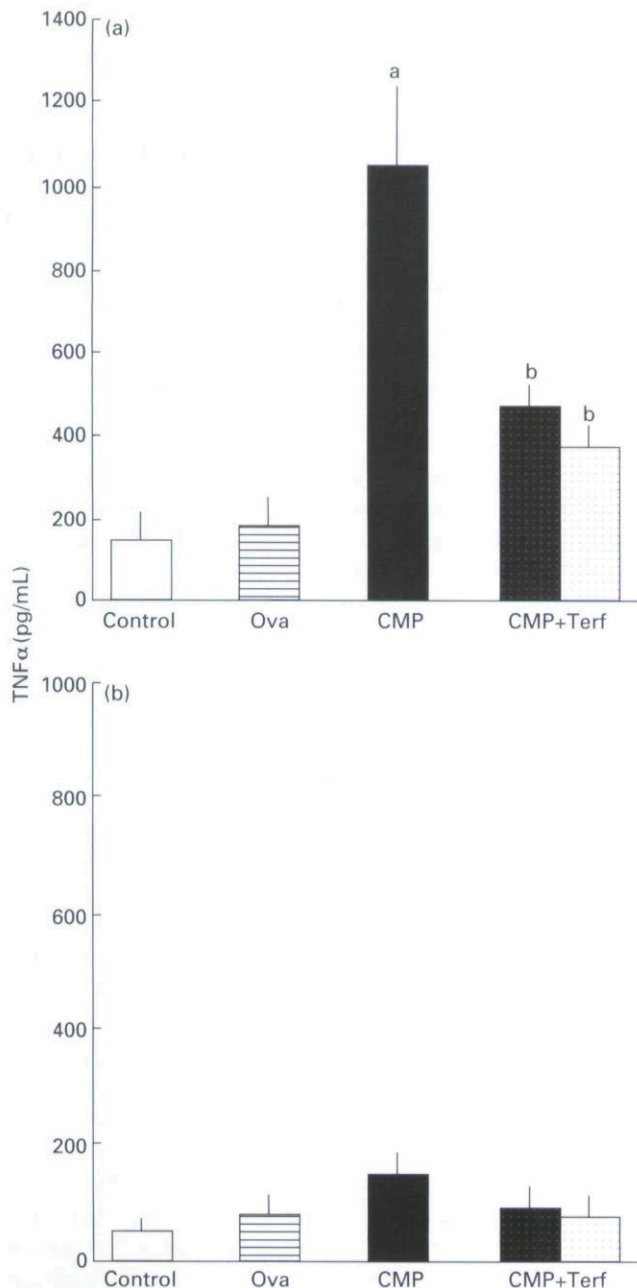


Fig. 1. Effect of terfenadine on PBMC viability. The effect of the vehicle of terfenadine (ethanol) as well as the effect of terfenadine alone, on LDH release (a) and cell viability (b) are reported. No significant toxicity is observed, except at the highest terfenadine concentration, 10 μ M. a, significantly different from all other conditions $P \leq 0.0001$; b, significantly different from Control $P \leq 0.04$. $n = 4$ –12 separate experiments. ■, Terf-LDH; □, Trifon-LDH.

increase in LDH release was observed in the presence of $10\text{ }\mu\text{M}$ terfenadine, suggesting that at this high concentration terfenadine damages the cell membranes. By contrast, neither ethanol nor terfenadine 0.1 or $1\text{ }\mu\text{M}$ had a deleterious effect on PBMC viability.

The same pattern was obtained when the number of viable cells was considered as indicated in Fig. 1B. There was a small and non-statistically significant decrease in cell number in the presence of ethanol, terfenadine 0.1 and $1\text{ }\mu\text{M}$, but in the highest terfenadine concentration a significant decrease in viable PBMC was observed. Therefore,



0.1 and $1\text{ }\mu\text{M}$ terfenadine concentrations were used in the study.

Effect of terfenadine on TNFα release from PBMC As shown in Fig. 2 (a) depicting the release of TNFα by PBMC from infants diagnosed as being still allergic to CMP (Active CMA group), basal TNFα secretion in the absence of stimulation was small. Stimulation with the non-milk antigen ovalbumin failed to enhance TNFα secretion. However, when PBMC were stimulated with cow's milk proteins (CMP) at a final concentration of $600\text{ }\mu\text{g/mL}$, TNFα secretion increased significantly. Such TNFα secretion was not related to the age of the children studied (12 months in the 'cured CMA' group and 27 months in the 'active CMA' group), as no correlation was found between age and TNFα secretion (not shown). Stimulation with $600\text{ }\mu\text{g/mL}$ CMP in the presence of increasing amounts of terfenadine (0.1 and $1\text{ }\mu\text{M}$) significantly reduced this secretion. The percentages of inhibition of TNFα secretion were 47 and 62% at 0.1 and $1\text{ }\mu\text{M}$ terfenadine, respectively.

The release of TNFα by PBMC from infants finally diagnosed as having recovered from CMA (Fig. 2(b).) showed that unlike the PBMC from infants with active CMA, PBMC from cured CMA infants secreted small amounts of TNFα in the presence of $600\text{ }\mu\text{g/mL}$ CMP. This secretion was not different from basal secretion. Terfenadine slightly reduced the CMP-induced secretion but the reduction was not significant.

Effect of PBMC culture supernatants on intestinal barrier capacity

Figure 3 shows the effect of PBMC culture supernatants on

Fig. 2. (a) Effect of terfenadine on TNFα release by peripheral blood mononuclear cells from 10 infants with active cow's milk allergy and gastrointestinal symptoms. Peripheral mononuclear cells ($10^6/\text{mL}$) were stimulated with a mixture of cow's milk proteins ($600\text{ }\mu\text{g/mL}$) with increasing amounts of terfenadine or without terfenadine. In the absence of terfenadine, TNFα release was greater than in unstimulated cells (Control) or in cells stimulated with the non-milk antigen ovalbumin (Ova). In the presence of cow's milk proteins and terfenadine (CMP+Terf), TNFα secretion gradually diminished. a, significantly different from other conditions $P < 0.0001$; b, significantly different from control $P \leq 0.04$, $n = 10$ infants. (b) Effect of terfenadine on TNFα release by peripheral mononuclear blood cells from 13 infants who had recovered from cow's milk allergy. TNFα secretion was not significantly increased by cow's milk proteins. It remained similar to secretion under control conditions (Control and Ova) and lower than that observed in infants with active cow's milk allergy ($P < 0.001$). Terfenadine reduced this TNFα secretion slightly but not significantly. $n = 13$ infants.

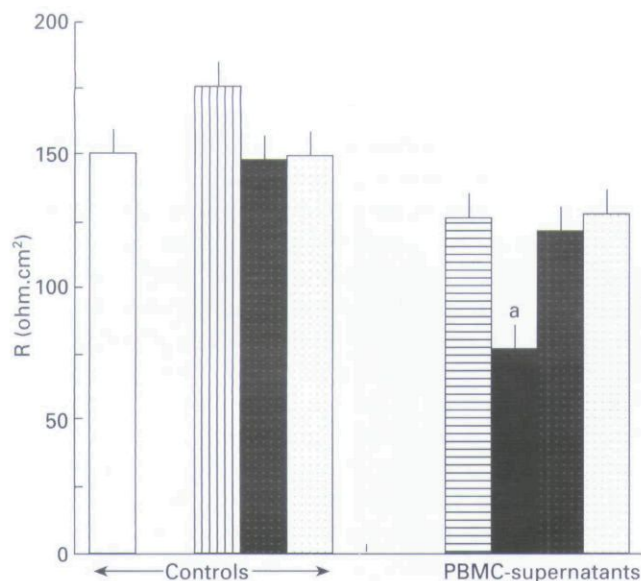


Fig. 3. Effect of peripheral mononuclear cell culture supernatants from eight infants (among them five tested twice) with active cow's milk allergy on the intestinal barrier capacity measured on the intestinal cell line HT29-19 A. The electrical resistance (R) was used as an index of intestinal integrity. Ethanol or terfenadine alone (Terf) tested at concentrations of 0.1 or 1 μ M (Controls) did not modify basal R (Basal), indicating that it had no direct effect on the intestinal cell line. Supernatants from peripheral blood mononuclear cells stimulated with cow's milk proteins in the absence of terfenadine (CMP), significantly reduced R compared with supernatants from unstimulated PBMC (Control). When terfenadine was added to cow's milk proteins (CMP+Terf), the supernatants did not induce any decrease in R. a, significantly different from Control and from (CMP+Terf) $P < 0.004$. $n = 9-21$ Filter-grown monolayers. □, Basal; ▨, Ethanol; ▩, Terf 0.1 μ M; ▤, Terf 1 μ M; ▭, Control; ■, CMP; ▧, CMP + Terf 0.1 μ M; ▨, CMP + Terf 1 μ M.

intestinal barrier capacity. We began by testing the direct effect of the vehicle of terfenadine (ethanol) and of 0.1 and 1 μ M terfenadine on the electrical resistance of intestinal HT29-19 A cell monolayers. In these initial experiments, neither ethanol nor 0.1 or 1 μ M terfenadine alone, modified significantly the basal electrical resistance (R), an index of epithelial integrity. This indicates that ethanol or terfenadine alone did not exert a toxic effect. Therefore, the cytotoxic effect of high terfenadine concentrations reported by some authors [7,10] was not observed in our intestinal cell line. In another set of experiments, we measured the effect on R of control PBMC culture supernatants and of supernatants containing CMP or CMP+ terfenadine. There was a significant decrease in the electrical resistance of HT29-19 A monolayers treated with supernatants containing CMP ($P < 0.004$), compared to the resistance of cells treated with control supernatants. In the presence supernatants

containing CMP+ terfenadine, this decrease was not observed, indicating that terfenadine had a protective effect.

Discussion

The present results confirm that during cow's milk allergy with intestinal symptoms, TNF α secretion rises, and that this rise alters intestinal barrier capacity. They further indicate that terfenadine greatly inhibits TNF α release by PBMC and therefore preserves the integrity of intestinal HT29-19 A cells, as measured *in vitro*.

Indeed, in a recent study, we reported that when PBMC from infants with CMA displaying gastrointestinal symptoms, were specifically stimulated by CMP, they secreted large amounts of TNF α thus altering intestinal permeability *in vitro* [12]. Subsequent results indicated that this alteration was due to the direct action of TNF α on tight junction complexes, leading to an increase in intestinal paracellular permeability [17]. This increase was constantly found *in vivo*, during cow's milk sensitive enteropathy using the dual lactulose/mannitol permeability test [18].

Terfenadine is generally administered in allergic diseases affecting the skin (eczema and atopic disease), airways (rhinitis) and eyes (conjunctivitis). In the skin diseases, it reduces the symptoms of the immediate allergic reactions and decreases both the early and late inflammatory cutaneous responses [2,10]. Terfenadine also inhibits the release of mediators from several cells. In mast cells, this inhibition seems to be due to the blockage of the calcium influx, as reported by Tasaka *et al.* [1] and might be related to its membrane-stabilizing action.

As terfenadine had been described as a potent inhibitor of various inflammatory mediators, we tested its ability to reduce TNF α secretion during CMA, and consequently, the deleterious effect of TNF α on intestinal barrier capacity.

CMA is a common disease whose pathophysiology is still poorly understood. Although IgE and mast cell-dependent mechanisms are observed, high IgE levels are not predominant in infants with CMA who display gastrointestinal symptoms, and T cell-mediated delayed reactions are suspected [19-21]. The suspicion was recently strengthened by our observation that TNF α makes an important contribution to the pathomechanism of CMA [12]. The present study confirms this contribution, and indicates that TNF α secretion is specific to cow's milk proteins, since it is not triggered by the non-milk related protein, ovalbumin.

TNF α is a proinflammatory mediator secreted by different cell types, including macrophages, basophils, mast cells [22] and eosinophils [23-25]. Although in the present study, we did not analyse the type of mononuclear cells responsible for TNF α secretion, we recently observed that these cells are probably monocytes/macrophages (i.e. adherent cells) whose specific stimulation needs the presence of

lymphocytes (non-adherent cells) [26]. The present work strengthens the hypothesis that mononuclear cells could be the target cells for terfenadine. This possibility was also suggested in a study of human lung cells comprising 60% monocytes, 28% lymphocytes and 6% mast cells [9].

In addition, terfenadine has been shown to impair the late phase cutaneous reaction [2,10] and T cells as well as T cell-derived cytokines have been implicated in this reaction [27]. Therefore, it seems more and more likely that terfenadine has the ability to stabilize a variety of immune cells, including monocytes/macrophages.

It is possible that the inhibition by terfenadine of $\text{TNF}\alpha$ release would have been even more pronounced *in vivo* than in the present *in vitro* observations.

Important inhibition, of up to 62%, occurred here *in vitro*, at terfenadine concentrations that might be found *in vivo* in the intestinal mucosa, since local intestinal concentration is likely to be higher than the serum concentration of terfenadine, which is very low (nM magnitude) after a first pass hepatic metabolism [28,29]. In the case of CMA with gastrointestinal symptoms, where the intestine is the target organ, terfenadine might concentrate in the lamina propria and act locally, before the hepatic clearance.

It is possible that *in vivo*, its effect is even more pronounced than *in vitro*, since more cell types are likely to be involved and synergistic effects may occur. For example, the inhibition of substance P by terfenadine [4] probably reduces $\text{TNF}\alpha$ release, because we know that substance P induces $\text{TNF}\alpha$ expression [30] and release [31] from human blood monocytes. Correlatively, the inhibition of $\text{TNF}\alpha$ secretion may prevent prostaglandin E2 release and the subsequent chloride secretion responsible for diarrhoea [32]. In addition, terfenadine has been shown to reduce ICAM-1 expression by epithelial cells [11]. Therefore, it might weaken lymphoepithelial interactions, either directly, by reducing ICAM-1 expression on intestinal epithelial cells, or indirectly, by inhibiting the secretion of inflammatory cytokines such as $\text{TNF}\alpha$, known to up-regulate ICAM-1 [33].

In conclusion, the present results indicate that terfenadine inhibits $\text{TNF}\alpha$ release by mononuclear cells from children with cow's milk allergy, *in vitro*. This inhibition prevents the harmful effect of $\text{TNF}\alpha$ on intestinal barrier capacity. Further studies are needed to determine whether such inhibition is found *in vivo*, and improves the clinical status of infants with intestinal symptoms of cow's milk allergy.

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