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Allergen-stimulated T lymphocytes from allergic patients induce vascular cell adhesion molecule-1 (VCAM-1) expression and IL-6 production by endothelial cells

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SUMMARY

Adhesion of inflammatory cells to endothelium is a critical step for their transvascular migration to inflammatory sites. To evaluate the relationship between T lymphocytes (TL) and vascular endothelium, supernatants from allergen-stimulated TL obtained from patients sensitive to *Dermatophagoides pteronyssinus* (*Dpt*) *versus* healthy subjects were added to endothelial cell (EC) cultures. TL were stimulated by autologous-activated antigen-presenting cells (APC) previously fixed in paraformaldehyde to prevent monokine secretion. Two parameters were measured: the expression of adhesion molecule and the production of IL-6. Related allergen-stimulated TL supernatants from allergic patients induced an increase of VCAM-1 and intercellular adhesion molecule-1 (ICAM-1) expression when supernatants of the control groups (TL exposed to an unrelated allergen or not stimulated or TL obtained from healthy subjects) did not. E-selectin expression was not modulated whatever the supernatant added to EC culture. IL-6 production by EC was significantly enhanced after activation with related allergen-stimulated TL supernatants from allergics compared with control supernatants. Induction of VCAM-1 expression was inhibited by adding neutralizing antibodies against IL-4, whereas IL-6 production and ICAM-1 expression were inhibited by anti-interferon-gamma (IFN-γ) antibodies. Enhanced production of IL-4 and IFN-γ was detected in related allergen-stimulated TL supernatants from allergic subjects compared with the different supernatants. These data suggest that allergen-specific TL present in the peripheral blood of allergic patients are of Th1 and Th2 subtypes. Their stimulation in allergic patients may lead to the activation of endothelial cells and thereby participate in leucocyte recruitment towards the inflammatory site.

Keywords T lymphocyte endothelial cell allergy cytokine

INTRODUCTION

Adhesion of peripheral blood cells to endothelium is a critical step for their further transendothelial migration toward sites of inflammation. Cell adhesion is achieved through the inter-action of cellular adhesion molecules, expressed by endothelial cells (EC), with their counterpart ligands expressed by leucocytes [1]. The expression of adhesion molecule is regulated by cytokines [2]. Tumour necrosis factor-alpha (TNF-α) and IL-1β, two monokines exerting pleiotropic effects, are the most potent cytokines able to up-regulate intercellular adhesion molecule-1 (ICAM-1) [3], E-selectin [4], and VCAM-1 [5–7] expression on endothelial cells. Lymphokines also up-regulate adhesion molecule expression, but in a more restricted manner. Interferon-gamma (IFN-γ) modulates ICAM-1 and HLA class II expression on endothelial cells. favouring mainly the adherence of neutrophils and T lymphocytes. IL-4, a lymphokine with a broad range of immune functions, up-regulates specifically VCAM-1 expression on endothelial cells, allowing a preferential eosinophil, basophil and T lymphocyte adhesion on these cells [8–10]. Thus, the expression of specific adhesion molecules may lead to the selection of leucocyte subpopulations. Following activation by proinflammatory cytokines, endothelial cells produce cytokines such as IL-1, IL-6, IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF) and may directly participate in the recruitment or activation of leucocytes [11].

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In this complex network, through lymphokine production, T lymphocytes are able to promote expression of cellular adhesion molecule and then to select cells susceptible to migrate further on. Several studies have focused on the evaluation of endothelial cell activation either *in vitro* by using human recombinant cytokines, or *ex vivo* by immunohistochemistry in several inflammatory diseases [12–14]. The inflammatory response in allergic disease is characterized by infiltrates of activated T lymphocytes and eosinophils [15]. Activated T lymphocytes express CD25 molecules and, in bronchial asthma, secrete lymphokines such as IL-3, IL-4, IL-5 or GM-CSF [16]. These lymphokines may be implicated, in combination with other proinflammatory cytokines (TNF-α and IL-1β) [17], in the development of chronic inflammation and bronchial hyper-reactivity. Thus, T lymphocytes appear to be one of the most important cells able to promote and regulate cellular events of the inflammatory reaction. We therefore looked at the activation of endothelial cells by T cell-derived lymphokines obtained after activation of peripheral blood T cells from allergic patients in the presence of the related allergen. For this purpose. T lymphocytes from allergic patients were stimulated by autologous antigen-presenting cells (APC) previously activated by the allergen, and fixed to block their own activation (i.e. monokine production).

MATERIALS AND METHODS

Allergens

Standardized allergens (Laboratoire des Stallergèns, Fresnes, France) were used in the study: *Detmatophagoides pteronyssinus* (*Dpt*) extract (100 IR/ml) (biological units used by the Laboratoire des Stallergènes) and grass-pollen extract (100 IR/ml), corresponding respectively to 140 and 35 μg/ml protein nitrogen. Hymenoptera venom was used as a control allergen.

Patients

Forty subjects (18–50 years old) were included in this study: 30 were allergic subjects and 10 were healthy donors without allergic disorders or atopy. Of the 30 allergic patients, 27 were sensitive to *Dpt* and presented asthma and/or perennial rhinitis, with usual clinical features of house dust mite sensitization; three patients were only sensitized to grass pollen. Thirteen were polysensitized and also presented clinical characteristics of grass pollen allergy. All patients presented positive cutaneous prick tests and exhibited class 2–4 positive radioallergosorbent test (RAST) (Pharmacia Biochemicals Inc., Uppsala, Sweden) (range from 6 to 52 RAST units). Healthy subjects had undetectable specific IgE against common aeroallergens. None of the subjects was sensitive to Hymenoptera venom, and patients did not receive any antiinflammatory therapy.

Peripheral blood mononuclear cells

Mononuclear leucocytes were prepared from 60 ml heparinized peripheral blood by density centrifugation on Ficoll–Hypaque (Pharmacia) [18]. After rosette formation with 2-amino ethylisothiouronium bromide-treated sheep erythrocytes, T lymphocytes were separated from B cells and monocytes (i.e. APC) by density sedimentation. After a further overnight incubation and the removal of the adherent cells, purity of the T lymphocyte suspension was assessed by immunofluorescence analysis using FITC-conjugated anti-human CD3 antibody (Becton Dickinson, Mountain View, CA) and was greater than 93% (data not shown). APC were adjusted at 5×10^6 cells/ml in culture medium consisting of RPMI 1640 medium complemented with 5% heat-inactivated fetal calf serum (FCS; GIBCO BRL, Cergy Pontoise, France), 100 U/ml penicillin, 10 μg/ml streptomycin and 2 mM L-glutamine. T lymphocytes were adjusted at 5×10^6 cells/ml in culture medium and cultured in 24-well cell culture plates (Nunc, Roskilde, Denmark) until use.

Stimulation of T lymphocytes

APC from allergic patients (5×10^6 cells/ml) were incubated in polypropylene tubes (Falcon) at 37°C in 5% CO_2 alone, with the related allergen (*Dpt* extract 10 IR/ml, or grass pollen extract 10 IR/ml), with an unrelated allergen (Hymenoptera venom 0·1 μg/ml) or alone. APC from healthy subjects were not stimulated or stimulated with 10 IR/ml *Dpt* extract. After 4 h of incubation. APC were fixed in 1% (v/v) paraformaldehyde (PFA) [19] in RPMI medium for 20 min at 37°C in 5% CO_2 with intermittent agitation. After extensive washing, APC were further incubated at 37°C for 20 h in culture medium, in order to saturate free sites of PFA and to discard unbound proteins.

PFA-fixed autologous activated APC ($0{\cdot}5 \times 10^6$) were added to 5×10^6 T lymphocytes/ml. After 24, 48 or 72 h of incubation, supernatants were collected, centrifuged at 1800 *g* for 10 min, aliquoted and stored at −80°C until use.

Activation of endothelial cells by T lymphocyte supernatants

Human endothelial cells were derived from umbilical vein, according to the method previously described [20,21]. Briefly, EC were collected after treatment of umbilical vein by 0·2% collagenase in RPMI medium for 15 min (GIBCO) and pelleted by centrifugation (1000 *g*, 10 min). After resuspension ($1{\cdot}2 \times 10^5$ cells/ml) in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 10 μg/ml streptomycin, 20% FCS (v/v), 100 μg/ml heparin, and 25 μg/ml endothelial cell growth supplement (Sigma Chemical Co, St Louis, MO), EC were cultured in 35-mm diameter tissue culture wells at 37°C in 5% CO_2. Endothelial cells were collected after trypsinization of confluent culture and cultured in gelatin-coated 96-well flat-bottomed plates until confluency. The EC were used at the third and fourth passages. T lymphocyte supernatants were added at 1 : 4 ratio to EC culture in a final volume of 100 μl and incubated for 10 h at 37°C. After incubation, EC culture supernatants were collected for the IL-6 quantification, aliquoted and stored at −80°C. EC were washed in 37°C prewarmed PBS and then fixed in 0·05% PBS/glutaraldehyde for 10 min at 4°C. After four washings in 0·1 M PBS pH 7·4 containing 5 mM EDTA and 5% (w/v) bovine serum albumin (BSA) (washing-assay buffer) and a further 1 h incubation in this buffer, the expression of adhesion molecules was analysed using specific ELISA. Positive controls consisted of optimal concentrations of rhTNF-α (200 U/ml) or rhIL-4 (200 U/ml) (Genzyme, Cambridge, MA).

Endothelial cell ELISA

The ELISA was performed on glutaraldehyde-fixed endothelial cells using the method originally described by Pober *et al.* [22]. Briefly, glutaraldehyde-fixed EC were incubated for 1 h with 100 μl/well of anti-VCAM-1 (clone BBIG-V1), anti-E-selectin (clone BBIG-E6; British Biotechnology Ltd, Oxford, UK), anti-ICAM-1 (clone 84H10) or control IgG1 MoAbs (Immunotech, Marseille-Luminy, France) at the experimentally

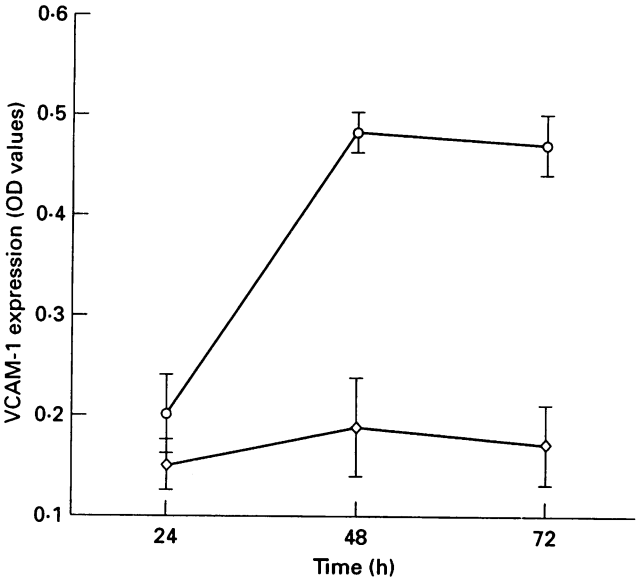

Fig. 1. Time course of lymphokine production inducing VCAM-1 expression on endothelial cells. T lymphocyte supernatants were obtained from five *Dermatophagoides pteronyssinus* (*Dpt*)-sensitive patients stimulated with the related allergen (○) or the unrelated allergen (◇). Results are expressed as mean ± s.d. of optical density (OD).

defined dose of 2 μg/ml. Cells were washed four times in washing-assay buffer and then incubated for 1 h with 100 μl/well of 1 : 5000 (v/v) diluted peroxidase-conjugated anti-mouse IgG rabbit antiserum (Pasteur Production, Courbevoie, France) in washing-assay buffer. After four washings, bound IgG were detected by addition of 100 μl 0·1 M citrate buffer pH 5·0 containing *o*-phenylenediamine and H_2O_2. Colour development reaction was stopped by addition of 100 μl/well 4 N HCl. Optical density (OD) was read at 492 nm and results were expressed as OD values.

IL-6 biological assay

Quantification of IL-6 production was evaluated in EC culture supernatants obtained 10 h after addition of 2 day-cultured T lymphocyte supernatants. IL-6 levels were assayed according to the technique described by Van Snick *et al.* [23] with some modifications. Briefly, the hybridoma cells 7TD1 (a generous gift of Dr Van Snick, Institut Ludwig, Brussels, Belgium) were incubated with serial dilutions of T lymphocyte supernatants in microtitre plates. The number of cells was evaluated 3 days later by a colorimetric assay. A 4 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT; Sigma) was added at 10%. After 4 h of incubation, the supernatants were discarded and 0·2 ml of 0·04 N HCl-isopropanol solution was added to each well to solubilize the reduced MTT precipitate. After homogenization, OD was read at 570 nm with a reference at 650 nm. All analyses were performed in duplicate and concentrations were calculated by a probit analysis in comparison with a standard of hrIL-6 (Boehringer, Mannheim, Germany). The results were expressed for each subject as percentage of increase in IL-6 production (defined in U/ml) as follows: ((La − Lo)/Lo) × 100, where La is the IL-6 level produced by EC activated with allergen-stimulated T lymphocyte supernatants, and Lo the IL-6 level produced by EC in the presence of non-stimulated T lymphocyte supernatants. The specificity of the test was controlled by the inhibition of cell proliferation after addition of a neutralizing anti-IL-6 rabbit antibody (Genzyme): in all cases, specific antibody induced an inhibition > 90%. Positive controls of EC activation were obtained with optimal concentrations of hrTNF-α (200 U/ml).

TNF biological assay

TNF-α concentration was evaluated in fixed-APC and T lymphocyte culture supernatants by L929 cytotoxic test. Biological activity was estimated from a standard curve as the amount necessary to kill 50% of actinomycin-treated L929 mouse fibroblasts after 18 h of culture. All analyses were performed in duplicate and concentrations were calculated by a probit analysis in comparison with a standard of hrTNF-α (Genzyme). The specificity of the test was controlled by the inhibition of cell killing after addition of a neutralizing anti-TNF-α rabbit antibody (Genzyme): in all cases, specific antibody addition induced inhibition > 90%.

IL-4 and IFN-γ quantification in T lymphocyte supernatants

Levels of IL-4 and IFN-γ were determined in T lymphocyte supernatants using commercialized kits (respectively from R&D Systems, Minneapolis, MN, and Genzyme). Results were expressed in pg/ml.

Statistical analysis

Results were expressed as mean ± s.e.m., except when mean ± s.d. was reported as precise. Statistical analysis was performed using the Wilcoxon test or the Mann–Whitney *U*-test.

RESULTS

Allergen-stimulated T lymphocytes from allergic patients induce VCAM-1 expression on EC

In preliminary experiments, a kinetic study was performed with cells from five *Dpt*-sensitive patients, in order to define the optimal timing of lymphokine production by allergen-stimulated T lymphocytes leading to a detectable modulation of VCAM-1 expression. Results showed that the highest expression of VCAM-1 was reached with allergen-stimulated T lymphocyte supernatants collected after 48 h culture (0·485 ± 0·02 OD (mean ± s.d.)) by comparison with those collected at 24 h (0·201 ± 0·04 OD). This expression remained elevated with supernatants collected at 72 h (0·472 ± 0·03 OD) (Fig. 1). Supernatants of T lymphocytes incubated with unrelated allergens did not modulate VCAM-1 expression.

Results of OD values corresponding to ICAM-1, E-selectin and VCAM-1 expression on EC obtained in presence of T lymphocyte supernatants collected after 48 h culture are presented in Table 1. Supernatants from non-stimulated T lymphocytes (TL) enhanced the expression of ICAM-1, E-selectin and VCAM-1 on EC (Table 1) compared with the level in untreated cells (0·307 ± 0·087, 0·096 ± 0·051, 0·085 ± 0·026) although the difference was not significant. In addition, there was no difference between the effect of non-stimulated TL from healthy subjects and allergic patients (P = NS).

Concerning VCAM-1 expression, OD values obtained with related allergen-stimulated TL supernatants from allergic patients (0·322 ± 0·07) were significantly higher than those obtained with cells exposed to an unrelated allergen

Table 1. Optical density for intercellular adhesion molecule-1 (ICAM-1), E-selectin and VCAM-1 expression on endothelial cells induced by T lymphocyte supernatants from allergic patients ($n = 30$) at baseline or stimulated with the related allergen or the unrelated allergen, compared with those obtained with T lymphocyte supernatants from healthy subjects ($n = 10$) at baseline or stimulated with allergen (mean ± s.e.m.)

	T lymphocyte supernatants obtained from				
	Allergic patients ($n = 30$)			Healthy subjects ($n = 10$)	
	Non-stimulated	Stimulated with the related allergen	Stimulated with the unrelated allergen	Non-stimulated	Stimulated with an allergen
ICAM-1	0·465 ± 0·09	0·554 ± 0·09*	0·487 ± 0·09	0·572 ± 0·03	0·592 ± 0·03
E-selectin	0·239 ± 0·02	0·270 ± 0·03	0·244 ± 0·02	0·261 ± 0·04	0·280 ± 0·05
VCAM-1	0·212 ± 0·03	0·322 ± 0·07*	0·227 ± 0·09	0·213 ± 0·10	0·203 ± 0·10

*$P < 0·05$ in comparison with the optical density obtained with supernatants from non-stimulated T cells. Supernatants of T lymphocytes were collected after a 48-h culture.

(0·227 ± 0·09), than with non-stimulated TL supernatants (0·212 ± 0·03) ($P < 0·03$) and than with allergen- or non-stimulated TL supernatants from healthy subjects (respectively 0·203 ± 0·1 and 0·213 ± 0·103; $P < 0·03$). For healthy subjects, supernatants from allergen-stimulated TL did not increase adhesion molecule expression on EC compared with unstimulated lymphocytes. As positive controls, 200 U/ml TNF-α- and 200 U/ml IL-4-activated EC ($n = 5$) gave OD values of 0·404 ± 0·042 and 0·239 ± 0·046, respectively, compared with OD values obtained on resting EC (0·085 ± 0·026).

Concerning ICAM-1 expression, OD values obtained with related allergen-stimulated TL supernatants from allergic patients (0·554 ± 0·29) were significantly higher than those obtained with an unrelated allergen (0·487 ± 0·09), and with non-stimulated TL supernatants (0·465 ± 0·09) ($P < 0·03$). In healthy subjects, supernatants from allergen-stimulated TL did not modulate ICAM-1 expression compared with non-stimulated TL supernatants (Table 1) (P = NS). As positive controls, 200 U/ml TNF-α-activated EC ($n = 5$) gave OD values of 0·869 ± 0·05, compared with OD values obtained on resting EC (0·307 ± 0·087).

In contrast, allergen-dependent stimulation of TL from healthy subjects and allergic patients did not modulate E-selectin expression on EC compared with non-stimulated TL supernatants (Table 1). Basal levels of E-selectin expression were 0·096 ± 0·051 and 0·350 ± 0·08 after activation of EC with 200 U/ml TNF-α.

Fixed-APC supernatants did not induce any modulation of these two cellular adhesion molecule expressions on endothelial cells. OD value obtained with the IgG1 control MoAb was 0·08 ± 0·04.

Allergen stimulation of T lymphocytes from allergic patients induces IL-6 production by endothelial cells

IL-6 production was not detected in allergen-stimulated or unstimulated T lymphocyte supernatants. Although IL-6 production by EC after activation by related allergen-stimulated TL supernatants (707 ± 192 U/ml (mean ± s.e.m.)) was higher than that obtained with unrelated allergen- or non-stimulated TL supernatants from 20 *Dpt*-sensitive patients (459 ± 168 U/ml and 410 ± 117 U/ml, respectively), the difference was not significant. Stimulation by allergen of T lymphocytes from healthy subjects did not increase significantly IL-6 production by EC compared with the level obtained with non-stimulated T cells (respectively 505 ± 66 U/ml and 547 ± 80 U/ml). However, T lymphocytes from healthy subjects and allergic patients produced spontaneously factor(s) enhancing significantly ($P < 0·05$) IL-6 production compared with the level secreted by untreated EC (90 ± 20·1 U/ml).

When IL-6 levels were expressed as an increased percentage of IL-6 production, T lymphocyte supernatants derived from *Dpt*- and/or grass pollen-sensitive patients stimulated by related allergen-activated APC augmented significantly the production of IL-6 by EC compared with the percentage obtained with supernatants from unrelated allergen-stimulated T lymphocytes (81 ± 7·9% compared with 5±7%, mean ± s.d.) ($P < 0·001$) (Fig. 2). Moreover, allergen-stimulated TL from healthy subjects did not increase IL-6 production (8 ± 6%).

TNF-α and IFN-γ activation of EC ($n = 6$) amplified also IL-6 production of 86 ± 12% and 65 ± 13%, respectively (mean ± s.d.) ($P < 0·05$). IL-4 activation did not modulate IL-6 synthesis.

IL-4 and IFN-γ appeared as the main T lymphocyte-derived mediators inducing activation of endothelial cells

In order to identify the nature of TL-derived factors that modulated VCAM-1 and ICAM-1 expression and IL-6 production, allergen-stimulated TL supernatants obtained from five allergic patients were preincubated with neutralizing polyclonal antibodies directed against IL-4, IFN-γ or TNF-α. The efficiency of antisera was evidenced by the neutralization of optimal activating doses of the corresponding human recombinant cytokines. Anti-TNF-α antibody did not inhibit any of these parameters (data not shown). Moreover, TNF-α was undetectable in supernatants of all allergen-stimulated TL collected at 48 h and of fixed APC. In contrast, VCAM-1 expression induced by allergen-stimulated TL was significantly inhibited in all cases by anti-IL-4 (mean of 85% of inhibition) ($P < 0·05$). In two out of five cases, anti-IFN-γ antibodies induced an inhibition of VCAM-1 expression (mean of 58% of inhibition) (Fig. 3). ICAM-1 expression and IL-6 production by EC were inhibited by anti-IFN-γ antibody (respectively, mean of 75% and 95% inhibition) ($P < 0·05$) (Fig. 3).

The concentrations of IL-4 and IFN-γ were determined in

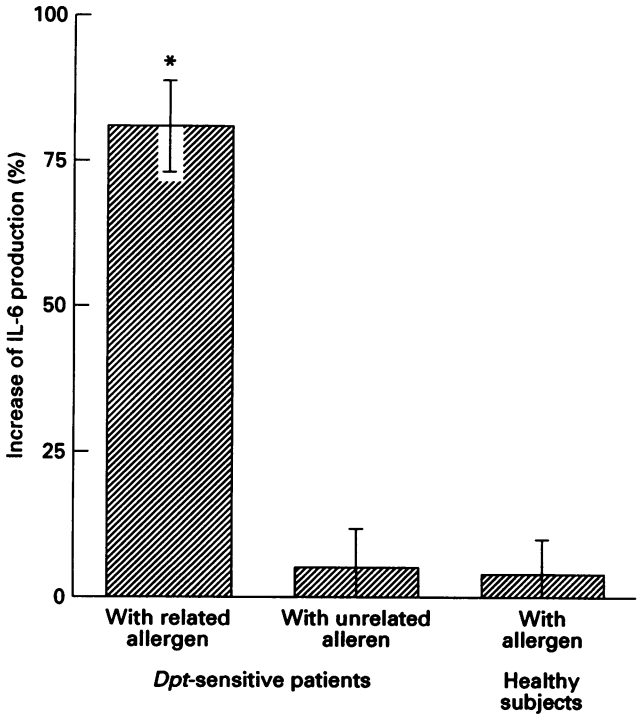

Fig. 2. Enhancement of IL-6 production by endothelial cells induced by related or unrelated allergen-stimulated T lymphocyte supernatants from allergic patients, or antigen-stimulated T lymphocyte supernatants from healthy subjects. Results are expressed as percentage of increase compared with the IL-6 production obtained with non-stimulated T cells. $*P < 0.01$ in comparison with the percentage obtained with supernatants from unrelated allergen-stimulated T lymphocytes. *Dpt, Dermatophagoides pteronyssinus.*

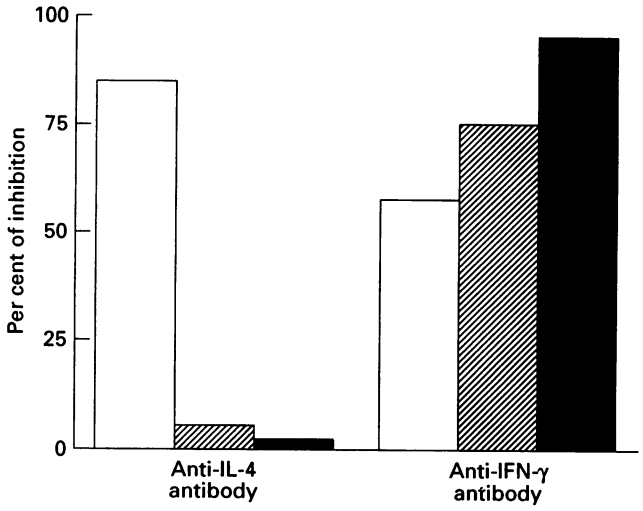

Fig. 3. Inhibition of VCAM-1 (□) and ICAM-1 expression (▨) or IL-6 production (■) by endothelial cells induced by allergen-stimulated T lymphocyte supernatants from allergic patients preincubated with anti-IL-4 or anti-IFN-γ neutralizing antibodies. Results are expressed as percentage of inhibition of VCAM-1 expression or IL-6 production (mean ± s.d.).

TL supernatants (Table 2). IL-4 production by related allergen-stimulated TL from allergic patients was significantly higher than that produced by non-stimulated or unrelated allergen-stimulated T lymphocytes ($P < 0.06$). IL-4 production by TL from healthy subjects was not modulated by stimulation with allergen (P = NS).

In contrast with the production of IL-4, IFN-γ production by allergen-stimulated T lymphocytes from allergic patients as well as from healthy subjects was significantly higher than the production by non-stimulated T lymphocytes (Table 2, $P < 0.05$). Similarly, activation of TL from allergic patients by unrelated allergen augmented significantly the production of IFN-γ in comparison with non-stimulated cells ($P < 0.05$).

DISCUSSION

Until now, the modulation of EC activation has been mainly studied using direct addition of human recombinant lymphokines on EC [2]. We therefore wanted to test the capacity of circulating T lymphocytes, recovered from allergic patients, to modulate directly the behaviour of the vascular endothelium, especially after triggering lymphocytes by specific allergens. Two parameters of EC activation were concomitantly evaluated: expression of cellular adhesion molecules, and the capacity to generate cytokines, and in particular one of the most heavily produced by EC, i.e. IL-6. The results demonstrated that peripheral blood T lymphocytes from allergic patients, stimulated by related allergen-primed autologous APC, induced enhanced expression of VCAM-1 and ICAM-1 and the production of IL-6 by EC. The mediators of this EC activation appeared to be IL-4 (and to a lesser extent IFN-γ) for VCAM-1 expression, and IFN-γ for IL-6 production and ICAM-1 expression.

To study the *in vitro* effects of T cells in response to an allergen stimulation, it was essential to avoid monokine secretion by APC in an appropriate manner that still allowed efficient antigen presentation to T lymphocytes [24–26]. Indeed, it is well known that monokines activate EC (increase in adhesion molecule expression and production of various inflammatory mediators). Moreover, after antigen presentation by PFA-fixed APC, a part of IL-1 expressed on the surface of fixed APC [24] is delivered in the medium at 12–24 h, and may interfere in EC activation [27]. Thus, in this study, after 20 h of culture, fixed APC were extensively washed before co-culture with T lymphocytes. In these experimental conditions, TNF-α was not detected either in fixed APC or in TL culture supernatants. Supernatants from fixed APC did not modulate adhesion molecule expression on EC. Moreover, as we did not observe any variation in the expression of E-selectin, we can hypothesize that the modifications observed in EC were only related to T lymphocyte products.

Inflammation is a characteristic feature of allergic diseases [13,28]. In allergic asthma, leucocyte infiltrate including eosinophils and activated TL is correlated with disease severity [29]. Leucocyte adherence to the endothelium is a prerequisite step for their further transmigration in tissues [1,30]. Although this process remains uncompletely understood, numerous cellular adhesion molecules involved in the adherence step are now defined (ICAM-1, VCAM-1 and E-selectin). The implication of adhesion molecules in the pathophysiology of asthma was demonstrated in primates by Wegner *et al.* [31] and Gundel *et*

Table 2. Production of IL-4 and IFN-γ by non-stimulated and allergen-stimulated T lymphocytes from healthy subjects ($n = 10$) and allergic patients ($n = 30$)

	Supernatants of T lymphocytes obtained from				
	Allergic patients			Healthy subjects	
	Non-stimulated	Stimulated with the related allergen	Stimulated with an unrelated allergen	Non-stimulated	Stimulated with an allergen
IL-4 (pg/ml)	8·9 ± 1·1	38·9 ± 2·1*	10·2 ± 1·5	9·9 ± 3·2	5·8 ± 2·8
IFN-γ (pg/ml)	117 ± 31	218 ± 25*	201 ± 26*	133 ± 41	207 ± 36*

Results are expressed in pg/ml (mean ± s.e.m.).
*$P < 0.05$ in comparison with the level obtained for non-stimulated cells.

al. [32], using neutralizing antibodies directed against ICAM-1 or E-selectin, able to inhibit the late-phase reaction and chronic bronchial hyperresponsiveness. Among these adhesion molecules, only VCAM-1 specifically mediates adherence of eosinophils and TL to EC [10]. Increased expression of VCAM-1 is observed in tissues chronically inflamed [33] or after allergen challenge in cutaneous and nasal biopsies from allergic patients [14,34]. Indeed, VCAM-1 expression is induced by TNF-α [6], IL-1 [35] and lipopolysaccharide (LPS), and more specifically by IL-4 [8]. More recently, Moser *et al.* [36] showed that EC activation by IL-4 leads to the transvascular migration of eosinophil from allergic patients, but not from healthy subjects. Injection of IL-4 in an animal model of cutaneous late-phase reaction induces a T cell infiltrate, correlated with increased VCAM-1 expression on EC [37,38].

In this study, and in accordance with the previously mentioned reports, we observed that only supernatants from related allergen-stimulated TL from allergic patients, but not from healthy subjects, induced enhanced VCAM-1 and ICAM-1 expression. IL-4 was quantified in TL supernatants and only detected in supernatants inducing VCAM-1 expression. Moreover, the involvement of IL-4 in the induction of VCAM-1 expression was reinforced by the fact that neutralizing anti-IL-4 antibodies inhibited this expression. The expression of ICAM-1 was increased by an IFN-γ-dependent mechanism.

In contrast, E-selectin was weakly modulated according to stimulatory conditions. Previous studies showed that cutaneous allergen challenge induced *in vivo* ICAM-1 and E-selectin expression on EC [12]. It could be argued that the expression of E-selectin in allergic reaction was essentially a consequence of resident cell activation (mastocytes and macrophages) rather than a consequence of T lymphocyte stimulation by relevant allergen. However, the spontaneous production of IFN-γ by T lymphocytes might be implicated in the effect of these supernatants on E-selectin expression by EC, since this cytokine induces it *in vitro* [11].

The second parameter by which we evaluated the activation of EC was production of IL-6. IL-6 is a pleiotropic cytokine produced by numerous cell types. Potential involvement of IL-6 in allergic response comprises [39–41]: (i) induction of B cell proliferation and differentiation; (ii) potentiation of IL-4-dependent IgE synthesis; and (iii) production of acute-phase proteins. Concerning the vascular endothelium, IL-6 induces vasopermeability but does not cause any infiltrate. IL-6 is produced by EC under activation by various stimuli such as TNF-α, IL-1, LPS and IFN-γ [12].

Since it was previously demonstrated that IFN-γ induced IL-6 production [11], we quantified this lymphokine in TL supernatants. IFN-γ was detected in related allergen-stimulated TL supernatants from allergic patients, and its involvement in IL-6 production was confirmed by the fact that anti-IFN-γ neutralizing antibodies inhibited IL-6 production. Nevertheless, IFN-γ was also detected in supernatants of TL from healthy subjects stimulated by antigen, and from allergic subjects stimulated with an unrelated allergen, whereas these supernatants did not modulate IL-6 production in comparison with non-stimulated TL. So the discrepancy observed between the presence of IFN-γ in these TL supernatants and the absence of effect on IL-6 production by EC suggests either (i) the production of a regulatory cytokine able to amplify in TL from allergic patients, IFN-γ effects on endothelial cells, or (ii) the production by stimulated T lymphocytes of an inhibitor of IFN-γ, particularly for healthy subjects exposed to allergen and for allergic patients in presence of an unrelated allergen. Further investigations are required to evaluate each of these hypotheses. Non-stimulated T cells from both groups induced a marked increase in IL-6 production by EC. The spontaneous secretion of IFN-γ by T cells at a similar level in healthy subjects and allergic patients may explain the effect of these supernatants.

IL-4 is produced by Th2-like T cell subtype (producing also IL-3, IL-5, and GM-CSF), while Th1-like T cells produce IL-2 and IFN-γ. While this profile was primarily described in the mouse, this dichotomy of TL subsets remains unclear in humans. This observation is in agreement with a previous study showing that T cell clones derived from allergic patients present a Th1- or Th2-like profile of lymphokine production in response to allergen stimulation [42]. This observation suggests a differently oriented response toward the environmental allergen in allergic patients compared with healthy subjects. Moreover, these Th2-like T cell subtypes constitute the majority of TL that infiltrate bronchial tissue [16]. In contrast, this study showed that allergen-stimulated peripheral TL from allergic patients are able to produce IL-4 and IFN-γ, while allergen-stimulated TL from healthy subjects produced only IFN-γ.

In conclusion, these results suggest that allergen-specific T cells may be: (i) of Th1 subtype in healthy subjects; and (ii) of

Th1 and Th2 or Th0 subtype in peripheral blood of allergic patients. The stimulation by allergen of these T lymphocytes from allergic patients leads to the production of lymphokines able to activate endothelial cells and then regulate the initiation and development of the inflammatory reaction.

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