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Modulation of adhesion molecule expression on endothelial cells during the late asthmatic reaction: role of macrophage-derived tumour necrosis factor-alpha

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SUMMARY

In a previous work we have demonstrated that in patients exhibiting a late allergic reaction (LAR), alveolar macrophages (AM) collected 18 h after bronchial allergen challenge produced high levels of IL-6 and tumour necrosis factor-alpha (TNF) which is known to up-regulate the endothelial cell expression of adhesion molecules participating in the development of the inflammatory reaction in bronchial asthma. For these reasons, we evaluated the effect of AM supernatants from asthmatic patients developing an LAR on intercellular adhesion molecule-1 (ICAM-1) and endothelial leucocyte adhesion molecule-1 (ELAM-1) expression by human endothelial cells. The expression of adhesion molecules was assessed by an ELISA method and compared with the effect of an optimal dose of human recombinant (hr) TNF. Results showed that AM supernatants, from challenged asthmatics developing an LAR, increased significantly the ICAM-1 and ELAM-1 expression on endothelial cells to a level similar to that obtained in the presence of hrTNF (500 U/ml) ($P < 0{\cdot}001$ in both cases, respectively 90·4% and 75·2% of the level obtained with hrTNF). In contrast, AM supernatants from asthmatics at baseline or exhibiting, after challenge, a single early reaction had no significant effect on these parameters (P = NS in both cases, respectively 23·5% and 24·7% of the ICAM-1 expression, 22·7% and 15·3% of the ELAM-1 expression obtained with hrTNF). AM-derived TNF present in these supernatants was thought to play a key role in endothelial cell stimulation, since: (i) TNF concentration in AM supernatants correlated with its effect on ICAM-1 ($r = 0{\cdot}80$, $P < 10^{-4}$) and ELAM-1 expression ($r = 0{\cdot}88$, $P < 10^{-5}$); and (ii) a neutralizing anti-TNF antibody decreased their effect (68% and 80% respectively on ICAM-1 and ELAM-1 expression). Moreover, the role of IL-6 was excluded on the basis both of the hrIL-6 inefficiency to induce ICAM-1 and ELAM-1 synthesis, even in costimulation with hrTNF, and of anti-IL-6 antibody to neutralize the effect of AM supernatants. Our results suggest that, beside mast cells and lymphocytes, macrophages might participate in the induction of the local inflammatory reaction observed in bronchial asthma. During the LAR, cytokines and especially TNF are able, through an enhanced adhesion molecule expression on endothelial cells, to facilitate the bronchial cellular influx.

Keywords late asthmatic reaction alveolar macrophages endothelium ICAM-1 ELAM-1 tumour necrosis factor

INTRODUCTION

It is now widely recognized that beside mast cells, other cells bearing IgE receptors including macrophages participate in the pathogenesis of allergic asthma [1,2]. In asthmatic patients, alveolar macrophages (AM) stimulated with anti-IgE or the related allergen release lysosomal enzymes, superoxide anion [3], a chemotactic factor for neutrophils and eosinophils [4] later related to leucotriene B4 [5], and monokines such as tumour necrosis factor-alpha (TNF) and IL-6 [6]. Furthermore, intra-bronchial instillation of an allergen in asthmatic patients rapidly induced *in situ* AM stimulation, arguing for an *in vivo* AM activation during the first step of the provocation test [7]. To extend our knowledge about the pathogenesis of the late asthmatic reaction (LAR), we evaluated in a previous study the spontaneous secretion of proinflammatory cytokines by AM recovered from allergic asthmatic patients challenged with a specific allergen. An increased release of TNF and IL-6 by AM was observed in patients who developed a dual response with an LAR [8], while no increased secretion by AM was measured in patients who presented a single early reaction (EAR). Moreover, Ohkawara *et al.* showed that in human lung fragments,

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TNF was released after IgE-receptor triggering only by mast cells and macrophages [9]. These results suggest that beside mast cells, macrophages may be involved in the late and inflammatory components of allergic asthma, in part through the release of monokines.

On the other hand, recent evidence has indicated that microvascular leakage and vascular endothelial cell activation support exudation and inflammatory cell recruitment. It was shown that endothelial-leucocyte adhesion molecule-1 (ELAM-1) and intercellular adhesion molecule-1 (ICAM-1) were strongly increased on endothelial cells following an experimental allergen challenge [10–12], and in allergic cutaneous inflammation [13,14]. Furthermore, treatment with anti-ICAM-1 MoAbs has been shown to decrease eosinophil infiltration and bronchial hyperreactivity in an animal model of asthma [11]. These results indicate that vascular endothelial cell expression of ICAM-1, which mediates LFA-1-dependent adherence of leucocytes, in particular of eosinophils, may be implicated in the inflammatory events associated with allergic asthma.

Because expression of these adhesion molecules is known to be up-regulated by TNF [15] and because this mediator was released by AM following allergen challenge, we focused our attention on the possible AM-dependent regulation of ICAM-1 and ELAM-1 expression on human endothelial cells *in vitro*. The expression of adhesion molecules was measured after endothelial cell activation by supernatants of AM collected 20 h after bronchial allergen challenge in asthmatic patients.

MATERIALS AND METHODS

Antibodies

The anti-ICAM-1 and anti-CD18 MoAbs were purchased from Immunotech (Marseille-Luminy, France); the anti-ELAM-1 and anti-PECAM-1 (anti-platelet endothelial cell adhesion molecule, an antibody that recognizes a 130-kD cell surface molecule (CD31, belonging to the immunoglobulin superfamily) involved in endothelial cell–cell contact [16]) MoAbs were purchased from British Bio-Technology (Oxford, UK). The control MoAb was a gift from J. Khalife (Institut Pasteur, Lille, France). All these MoAbs were IgG1. The neutralizing rabbit anti-sera against IL-6 and TNF were purchased from Genzyme (Boston, MA) and the rabbit antiserum against interferon-gamma (IFN-γ) was kindly provided by J. Wietzerbin (Institut Curie, Paris, France). A peroxidase-labelled rabbit antiserum anti-mouse IgG was purchased from Institut Pasteur Production (Courbevoie, France).

Patients

Alveolar macrophages were obtained from the patients studied by Gosset *et al.* [8], including 25 asthmatic patients (asthmatics at baseline, $n=8$; challenged asthmatics with an isolated EAR, $n=10$; challenged asthmatics with an EAR followed by an LAR, $n=5$) and 10 asymptomatic control subjects. Two allergen extracts were used for bronchial challenge: *Dermatophagoïdes pteronyssinus* standardized in reactivity index units (RIU) or wheat flour extract quantified in weight per volume (both from Stallergènes, Fresnes, France). Increasing doses of allergens were successively given by inhalation through an electric nebulizer (Mediprom SDC 88, Paris, France). Forced expiratory manoeuvres were done 5 min and 10 min after each inhalation of allergen. The first allergen concentration producing a fall of 20% FEV_1 was considered as a positive EAR. After development of EAR, FEV_1 values were followed up to the tenth hour after the beginning of the provocation test: a second fall of FEV_1 higher than 20% was considered as reflecting an LAR. AM were recovered by bronchoalveolar lavage after instillation of physiological saline into the bronchoalveolar tree under fibreoptic bronchoscopy and then isolated as previously described [3,17]. In the case of patients submitted to bronchial allergen challenge, bronchoalveolar lavage was performed 18 h after the provocation test as previously described [8]. Briefly, lavage fluid was filtered through sterile surgical gauze and centrifuged at 400 ***g*** for 10 min at 4°C. After three washings, the pellet was resuspended at a cell concentration of $1{\cdot}5 \times 10^6$/ml in RPMI 1640 containing 5% heat inactivated fetal calf serum (FCS) and 2 mM L-glutamine (GIBCO, Cergy Pontoise, France). Endotoxin contamination of medium was controlled by limulus amoebocyte test (Coatest, Kabevitrum, Austria) and was below 50 pg/ml. Cells were allowed to adhere to plastic Petri dishes (2 ml in 35-mm diameter well) for 2 h at 37°C. The non-adherent cells were removed by three washings with RPMI. Adherent cells contained more than 95% AM and less than 1% lymphocytes. After a 24-h incubation at 37°C in humidified air with 5% CO_2, culture supernatants were harvested and filtered through 0·45-μm filters (Millipore, Molsheim, France).

Monokine assay

TNF and IL-6 concentrations were evaluated in AM supernatants by the following methods. TNF concentration was measured by a sandwich radioimmunoassay (IRE-Medgenix, Fleurus, Belgium) after adequate dilution, and IL-6 amounts were estimated by the proliferation test of 7TD1 cells (a generous gift of Dr Van Snick, Institut Lüdwig, Brussels, Belgium) as described in the associated paper [8]. Specificity of the test was controlled by inhibition of cell proliferation with a neutralizing anti-IL-6 rabbit antibody, and the results were expressed in U/ml as defined by a standard of hrIL-6 (Boehringer-Mannheim, Mannheim, Germany). In all cases, specific antibody addition to AM supernatants induced an inhibition greater than 90%.

Human endothelial cell culture

Endothelial cells were derived from human umbilical vein as previously described [18] and cultured in endotoxin-free RPMI 1640 medium containing 20% heat-inactivated FCS, 100 μg/ml heparin and 25 μg/ml endothelial cell growth supplement (all reagents were purchased from Sigma). Endothelial cells were then subcultured at the second passage on gelatin-coated 96-microwell culture plates (Falcon, Becton Dickinson). Experiments were started at cell confluency.

Endothelial cell activation

Confluent endothelial cells were incubated with recombinant cytokines or with AM supernatants before the measurement of adhesion molecule expression. AM supernatants were added at 1:4 and 1:20 dilution in a final volume of 100 μl and incubated for 4 h or 24 h at 37°C (respectively for ELAM-1 and ICAM-1 expression). Controls included the addition of different concentrations of human recombinant TNF (hrTNF, from 10 to 500 U/ml) and IFN-γ (hrIFN-γ, from 10 to 500 U/ml) (these recombinant cytokines were kindly provided by Dr J. Wietzerbin, Institut Curie, Paris, France). In some experiments the

effect of rhIL-6 addition (200 and 2000 U/ml, Boehringer-Mannheim) was also evaluated on unstimulated endothelial cells or cells activated by hrTNF. Endothelial cells were then washed twice with PBS, fixed for 10 min at 4°C with 0·5% glutaraldehyde in PBS, washed four times with PBS containing 10^{-2} M EDTA and 0·1% bovine serum albumin (BSA), and incubated for 1 h with this same medium.

In order to identify the AM-derived inducer of adhesion molecule expression on endothelial cells, the supernatants or the recombinant cytokines were incubated with rabbit antisera to TNF, IL-6 and IFN-γ (all were used at 1:35 dilution) during 2 h at 37°C before addition to endothelial cell cultures. This concentration was theoretically sufficient to neutralize AM-derived cytokines as defined by the neutralizing activity of the antibodies. In addition. AM supernatants incubated with anti-TNF and anti-IL-6 were inactive in the respective bioassays.

Endothelial cell ELISA

The expression of adhesion molecules was measured by an ELISA method. This was performed on glutaraldehyde-fixed endothelial cells using the method described by Pober *et al.* [15,19], slightly modified. Briefly, fixed endothelial cells were incubated for 1 h with 100 μl/well of IgG1 MoAb to ICAM-1 and to CD18, to ELAM-1 and to PECAM-1 or the control IgG1 MoAb, at the experimentally defined dose of 0·4 μg/ml. Cells were washed again (four times) and incubated for another hour with 100 μl of 1:5000 (v:v) diluted peroxidase-labelled anti-mouse IgG rabbit antiserum. After four additional washings, 100 μl of substrate buffer containing H_2O_2 and *o*-phenylenediamine were added for 30 min, after which the reaction was stopped with 100 μl of 2 N H_2SO_4. Optical density (OD) was read in a multiwell scanning spectrophotometer at 492 nm. All analyses were performed in triplicate.

Results obtained with AM supernatants were expressed in some cases as the percentage of the OD obtained with hrTNF (500 U/ml) = (OD of AM supernatant − OD of medium alone) × 100/(OD of rhTNF − OD of medium alone). The inhibitory effect of neutralizing antibodies on adhesion molecule expression was expressed as the percentage = ((OD of activator plus antibody) − OD of medium alone) × 100/(OD of activator − OD of medium alone).

Statistical analysis

Statistical analysis was performed using either the Mann–Whitney *U*-test or the Wilcoxon test. The correlations were determined using the Spearman rank test. Results were expressed as mean ± s.d.

RESULTS

Endothelial cell adhesion molecule expression after addition of human recombinant cytokines

hrTNF induced both ICAM-1 and ELAM-1 expression on cultured human endothelial cells. While ICAM-1 expression reached its maximum at 24 h and remained elevated until 48 h after hrTNF stimulation, ELAM-1 expression peaked between 4 h and 6 h and then returned to the baseline at 24 h (Fig. 1). PECAM-1 (CD31) was expressed on the surface of endothelial cells in our culture conditions, but was not modulated by hrTNF, and was slightly inhibited 24 h after incubation with hrIFN-γ (Fig. 2). CD18 expression was undetectable even after

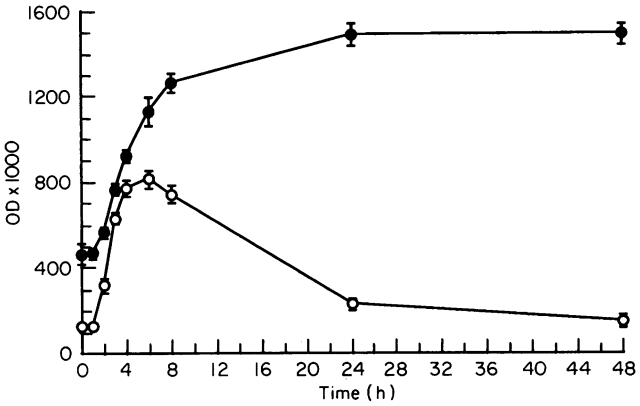

Fig. 1. Time-course of intercellular adhesion molecule-1 (ICAM-1) (●) and endothelial leucocyte adhesion molecule-1 (ELAM-1) (○) expression induced by 500 U/ml of hrTNF on cultured human endothelial cells. Data are expressed as mean optical density (OD) ± s.d. of three separate experiments.

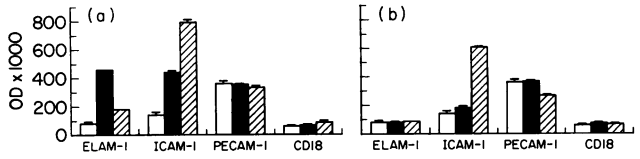

Fig. 2. Effect of hrTNF (500 U/ml (a)) or hrIFN-γ (500 U/ml (b)) on the expression by endothelial cells of various adhesion molecules. Cells were stimulated with the two cytokines either 4 h or 24 h before the evaluation of cell adhesion molecule expression. MoAbs were all used at a constant concentration of 0·04 μg/well. This figure represents one among four separate experiments. The threshold optical density was 0·062 ± 0·006. Data are expressed as mean of optical density (OD) ± s.d. (a) □, Medium; ■, hrTNF (4 h); ▩, hrTNF (24 h). (b) □, Medium; ■, hrIFN-γ (4 h); ▩, hrIFN-γ (24 h). ELAM-1, endothelial leucocyte adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; PECAM-1, platelet endothelial cell adhesion molecule-1.

stimulation with recombinant cytokines (Fig. 2). When stimulated by hrIFN-γ (500 U/ml), ICAM-1 expression increased at a magnitude similar to that induced by hrTNF (Fig. 2). In contrast, ELAM-1 expression was unaffected by hrIFN-γ (Fig. 2) as previously described [15]. The dose-response curves showed a plateau in ICAM-1 and ELAM-1 expression at doses up to 200 U/ml hrTNF and 500 U/ml hrIFN-γ (data not shown). In addition, hrIL-6 did not exhibit any activity on ICAM-1 and ELAM-1 expression, alone or in costimulation with hrTNF at different doses (Fig. 3).

Modulation of endothelial cell adhesion molecules after addition of supernatants from AM obtained in allergic asthmatics

Human endothelial cells were incubated in the presence of AM supernatants from asthmatic patients exposed to bronchial allergen challenge for 4 h and 24 h in order to appreciate the modulation of ELAM-1 and ICAM-1 respectively. AM supernatants derived from asthmatics who developed an LAR induced a significant increase in ICAM-1 and ELAM-1 expression ($P < 0·001$ in both cases, Fig. 4) at a level similar to that obtained with an optimal concentration of hrTNF. On the other

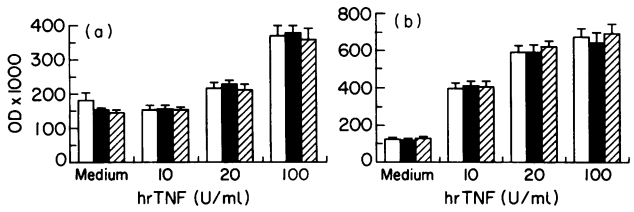

Fig. 3. Effect of hrIL-6 alone (200 (■) and 2000 U/ml (▩)) or with different doses of hrTNF (10, 20 and 100 U/ml) on intercellular adhesion molecule-1 (ICAM-1) (a) and endothelial leucocyte adhesion molecule-1 (ELAM-1) (b) expression by endothelial cells. This figure shows one representative experiment out of three. Results are expressed as mean optical density (OD) ± s.d. □, No hrIL-6.

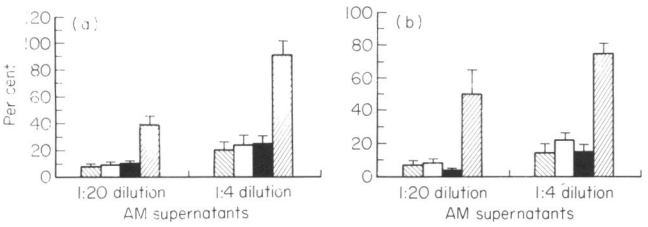

Fig. 4. Modulation of intercellular adhesion molecule-1 (ICAM-1) (a) and endothelial leucocyte adhesion molecule-1 (ELAM-1) (b) expression on endothelial cells by alveolar macrophage (AM) supernatants obtained from controls and from asthmatic patients at baseline or after bronchial challenge: patients exhibiting a single early allergic reaction (EAR) or an EAR followed by a late allergic reaction (LAR). Results are expressed as mean ± s.d. of percentage of optical density (OD) = ((OD of AM-supernatant − OD of medium)/(OD of hrTNF − OD of medium)) × 100. Means of OD obtained with medium alone and after addition of hrTNF (500 U/ml) were respectively: 0·361 ± 0·105, 0·955 ± 0·240 OD ($n = 5$) for ICAM-1, and 0·193 ± 0·043, 1·305 ± 0·285 OD ($n = 5$) for ELAM-1 expression. ▧, Controls; □, unchallenged; ■, EAR; ▩, LAR.

hand, AM supernatants from controls and from asthmatics at baseline or who exhibited a single EAR after challenge, increased slightly but not significantly ICAM-I and ELAM-1 expression (Fig. 4). The incubation time necessary to obtain maximal expression of ELAM-1 and ICAM-1 was similar to that observed with hrTNF.

Comparison of TNF and IL-6 amounts measured in AM supernatants with the AM-dependent ICAM-1 expression showed a close correlation between ICAM-1 and the cytokine levels (respectively $r = 0{\cdot}80$, $P < 10^{-4}$ and $r = 0{\cdot}61$, $P < 10^{-3}$). Indeed, the highest TNF and IL-6 concentrations were found in AM supernatants from challenged asthmatics with an LAR, whereas IL-1β concentrations were unchanged compared with the control subjects [8]. In addition, TNF and IL-6 concentrations in AM supernatants were closely correlated ($r = 0{\cdot}91$, $P < 0{\cdot}001$). Similarly, ELAM-1 expression induced by AM supernatants also correlated with TNF and IL-6 levels (respectively $r = 0{\cdot}88$, $P < 10^{-5}$ and $r = 0{\cdot}82$, $P < 10^{-4}$).

In experiments using neutralizing antibody directed against cytokines, only anti-TNF antibody could significantly inhibit AM-dependent ICAM-1 and ELAM-1 expression on endothelial cells ($P < 0{\cdot}01$ in both cases, Fig. 5), whereas anti-IFN-γ and anti-IL-6 antibodies were ineffective. The specificity was con-

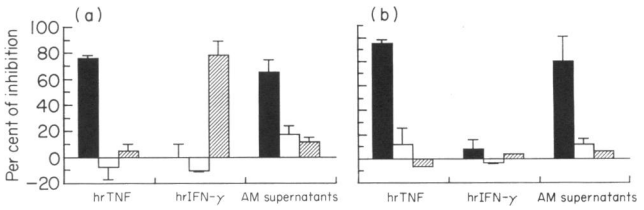

Fig. 5. Effect of different neutralizing antibodies on intercellular adhesion molecule-1 (ICAM-1) (a) and endothelial leucocyte adhesion molecule-1 (ELAM-1) (b) expression induced by hrTNF, hrIFN-γ (500 U/ml in both cases) and by the five alveolar macrophage (AM) supernatants (1:10 dilution) from patients with late allergic reactions (LAR). These experiments were repeated three times. Results are expressed as the percentage of inhibition obtained in the presence of the different antibodies compared with the inducer alone (respectively hrTNF, hrIFN-γ and AM supernatants). ■, Anti-TNF; □, anti-IL-6; ▩, anti-IFN-γ.

firmed by the fact that rhTNF and rhIFN-γ were neutralized by the specific antiserum only and not by the others. Moreover, the role of IL-6 was excluded on the basis of the inefficiency of anti-IL-6 anti-serum to neutralize the effect of AM supernatants (Fig. 5) whereas this antibody inhibited at 93% the IL-6 activity in the 7TD1 proliferation assay.

DISCUSSION

In this study, supernatants of AM retrieved from allergic asthmatics exhibiting an LAR after bronchial allergen challenge were shown to enhance adhesion molecule expression on cultured human endothelial cells. In a previous work, we demonstrated that AM recovered after an LAR secreted large amounts of TNF and IL-6. It was logical to test the capacity of AM supernatants to modulate the expression of adhesion molecules on potential target cells present in the bronchial mucosa, such as endothelial cells.

For this purpose, we chose to develop an ELISA method in order to evaluate the modulation of cell adhesion molecule expression in the presence of various recombinant human cytokines such as TNF, IFN-γ and IL-6. In this work, the different kinetics of ICAM-1 and ELAM-1 expression induced by rhTNF were similar to those reported elsewhere [15]. Moreover, the expression of CD18 and PECAM-1 antigen was undetectable either on unstimulated cells or on endothelial cells activated by cytokines. hrIFN-γ up-regulated ICAM-1 expression, whereas it had no effect on ELAM-1 expression. These observations, previously demonstrated using other methods, clearly show that our ELISA method is a useful tool to investigate the regulation of adhesion molecule expression on vascular endothelial cells.

Many of the characteristics of bronchial asthma can be explained on the basis of inflammatory processes induced by various mediators released after IgE–allergen interaction. Mast cell-derived products play a key role in the immediate bronchoconstriction, but the LAR seems to require additional proinflammatory components. The rapid influx of inflammatory cells (neutrophils and mainly eosinophils) [20–22], the release of cationic proteins [23] and the efficiency of corticosteroid treatment on the LAR [24] strongly suggest the participation of proinflammatory mediators in this process, among which monokines like TNF appear as an attractive hypothesis. Indeed,

TNF can activate eosinophils and neutrophils [25], whereas IL-6 has no known action on these cells. TNF can also amplify the generation by various cells of platelet activating factor [26], which is a very potent chemotactic factor for eosinophils [27].

In this context, the recruitment and subsequent activation of neutrophils and eosinophils represent a major event in the LAR. However, local conditions inducing adherence and transvascular migration of granulocytes toward the site of the allergen conflict are still incompletely defined. It has been reported recently that ELAM-1 expression on vascular endothelial cells from cutaneous tissue sections appeared 6 h after stimulation with allergen, anti-IgE or compound 48/80, and correlated with mast cell degranulation [10,14]. ELAM-1 expression appears to be TNF-dependent and can be inhibited by prior incubation with cromolyn, a drug able to prevent mast cell degranulation [10]. In a recent report, Walsh *et al.* demonstrated that mast cells were the main source of TNF in normal human dermis, and that their degranulation released TNF, which in turn directly induced ELAM-1 on dermal microvascular endothelium [28]. In the lung, the situation is quite different, since AM and at a lower level neutrophils are the main cells covering the epithelial cell surface as shown by Rankin *et al.* [29] and by Eschenbacher & Gravelyn [30]. Furthermore, TNF may also come from macrophages, as shown *in vitro* in an IgE-dependent pathway [6] and *ex vivo*, in LAR induced after bronchial allergen challenge [8]. Moreover, as described for mast cells, cromolyn inhibits IgE-dependent activation of AM [31]. These data and the recent observation made by Ohkawara *et al.* [9] show that both macrophages and mast cells present in the airways produce TNF in the lung after IgE-dependent triggering. These results may also be relevant to naturally occurring asthma, since high levels of TNF and other cytokines are found in bronchoalveolar lavage from atopic patients with symptomatic asthma compared with asymptomatic patients [32].

Although the precise role of AM *in vivo* is not defined, our present results indicate that, among AM-derived mediators, TNF is able to act on endothelial cells by increasing expression of ICAM-1 and ELAM-1, involved in eosinophil and neutrophil adherence [33]. It is noteworthy that a close correlation was observed between AM-dependent production of TNF, TNF-dependent increase of ICAM-1 and ELAM-1 on endothelial cells, and the development of the LAR *in vivo*. In contrast, secretion of IL-6, which was similarly enhanced during the LAR, did not appear to be relevant since anti-IL-6 antibody did not inhibit AM-derived ICAM-1 and ELAM-1 expression, and rhIL-6 did not increase these parameters. However, AM present in the bronchial lumen are not in close contact with endothelial cells, and it remains to be determined if TNF produced by these cells can reach the endothelium. These data obtained from patients with allergic asthma can be compared with results recently reported in the primate model. In these studies, after allergen challenge ICAM-1 and ELAM-1 were strongly expressed on vascular endothelial cells from lung biopsies [11,12]. ELAM-1 was often expressed 6 h following a single allergen challenge [12], while ICAM-1 was preferentially expressed following repeated challenges [11]. Furthermore, superfusion of MoAbs to ICAM-1 resulted in the decrease of both bronchial hyperreactivity and eosinophil infiltration, thus suggesting that expression of ICAM-1 on vascular endothelial cells may play a pivotal role in the inflammatory process of allergic asthma [11].

Finally, our results suggest that, beside mast cells, pulmonary macrophages can participate in the induction of the inflammatory reaction observed in asthma, through endothelial cell activation. Moreover, TNF seems to play a key role in the expression of endothelial cell adhesion molecules and the subsequent influx of inflammatory cells. These data also suggest that drugs that counteract TNF secretion and/or endothelial cell activation might be useful in the therapeutic management of allergic asthma.

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