

Gradual increase in priming of human eosinophils during extravasation from peripheral blood to the airways in response to allergen challenge

Bart Luijk, MD,^a Caroline A. Lindemans, MD,^a Deon Kanters, BSc,^a Roos van der Heijde, MD, PhD,^a Paul Bertics, PhD,^b Jan-Willem J. Lammers, MD, PhD,^a Mary-Ellen Bates, PhD,^b and Leo Koenderman, PhD^a *Utrecht, The Netherlands, and Madison, Wis*

Background: Eosinophils isolated from the blood of patients with allergic asthma exhibit enhanced responsiveness to multiple stimuli compared with cells from normal controls, a phenomenon generally referred to as *priming*. This priming response is essential for optimal activation with augmented responses including chemotaxis, cytotoxicity, respiratory burst, and the release of proinflammatory lipid mediators.

Objective: To monitor the kinetics of priming of eosinophils in the peripheral blood and in the bronchoalveolar lavage fluid of patients with allergic asthma before and after allergen challenge.

Methods: Priming of blood eosinophils obtained from patients with allergy and donors without allergy was measured by labeling with monoclonal phage antibodies A17 and A27 recognizing priming-associated epitopes on phagocytes. In addition, blood and bronchoalveolar lavage fluid eosinophils from subjects with allergy after segmental and whole lung allergen challenge were similarly analyzed.

Results: A dose-dependent cytokine-induced upregulation of priming-associated epitopes on blood eosinophils was found. Patients with allergic asthma exhibited an *in vivo* partially primed eosinophil phenotype, which is further primed *in vitro* after cytokine or chemokine incubation. Priming was increased in peripheral blood 6 hours after whole lung challenge as well as after segmental allergen challenge. Interestingly, eosinophils obtained from the bronchoalveolar lavage fluid 48 hours after segmental allergen challenge exhibited a higher primed phenotype.

Conclusion: These data are consistent with a model in which local allergic inflammatory reactions induce partial systemic eosinophil priming in the peripheral blood. Eosinophils found in the airway are highly primed, consistent with the

markedly upregulated inflammatory capacity observed in these cells. (*J Allergy Clin Immunol* 2005;115:997-1003.)

Key words: Priming, allergen challenge, eosinophils, allergic asthma, peripheral blood

Allergic asthma is accompanied by a chronic inflammatory reaction in the airways.¹ This inflammation is characterized by the presence of eosinophils and CD4⁺ T cells in the airway, and several lines of evidence indicate that this inflammatory reaction is an important determinant in allergic asthma.² These data were obtained by analyzing biopsies taken from the airways of patients with allergic asthma at different stages of this disease.³ Indeed, many studies using anti-inflammatory treatment have shown that the inflammatory reaction is an integral part of the pathogenesis of the disease.^{4,5} Treatment with inhaled glucocorticosteroids is the therapy of choice to inhibit inflammatory processes seen in the airways of patients with moderate to severe asthma.⁶

Evaluation of the extent of inflammation in the bronchial tissue is hampered by the required bronchoscopies that are tedious and expensive and impose a substantial discomfort on the patients. This has provoked many investigators to develop disease markers that can monitor this inflammatory reaction through less invasive methods. These methods range from the analysis of induced sputum to exhaled gases like nitric oxide and H₂O₂.^{7,8} Several approaches have also been developed to measure markers in peripheral blood such as serum eosinophilic cationic protein and soluble adhesion molecules.^{9,10} Several of these markers have proven to be applicable in population-based studies but appeared to have their limitations to monitor the disease using repeated measures in individual patients.

Several studies have shown that eosinophils from subjects with allergic asthma exhibit a primed phenotype likely as a consequence of their interaction with cytokines in peripheral blood, which facilitates extravasation to the airways with an increased chemotaxis and endothelial adhesion response.¹¹⁻¹⁴

Despite the recognition of the importance of eosinophil priming in the pathogenesis of allergic diseases, no antibodies have yet been described that can identify these primed eosinophils with a sufficient dynamic range to

From ^athe Department of Pulmonary Diseases, University Medical Center, Utrecht; and ^bthe Department of Biomolecular Chemistry, University of Wisconsin.

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Reprint requests: Leo Koenderman, PhD, Department of Pulmonary Diseases, University Medical Center, Heidelberglaan 100, NL 3584 CX Utrecht, The Netherlands. E-mail: L.Koenderman@hli.azu.nl.

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Abbreviations used

AU:	Arbitrary units
BAL:	Bronchoalveolar lavage
FITC:	Fluorescein isothiocyanate
FMLP:	N-formyl-methionyl-leucyl-phenylalanine
MCF:	Median channel fluorescence
MoPhab:	Monoclonal phage antibody
SBP:	Segmental bronchial provocation

allow detailed priming studies *in vivo* and *in vitro*. We recently developed 2 mAbs from a semisynthetic phage antibody library.¹⁵ These antibodies identified neutrophils and monocytes in blood primed *in vitro* with low doses of cytokines (picomolar range) and *in vivo* in patients with unstable chronic obstructive pulmonary disease.¹⁵ Here we studied the priming of eosinophils in allergic asthma both in tissue and in peripheral blood.

METHODS**Patients**

Thirty-eight patients who had mild asthma according to the definition of the Global Initiative for Asthma guidelines were selected from patients attending the outpatient clinic of the University Medical Center, Utrecht, The Netherlands, or via advertisements.⁶ All subjects had a history of episodic wheezing and periods of impaired lung function (Table I). Twenty-two of these patients with asthma underwent an inhaled allergen challenge according to a standardized protocol as described previously (for patient details, see the Journal's Online Repository at www.mosby.com/jaci).¹⁶ Healthy controls were selected from the laboratory and clinical staff without a history of asthma or presence of atopy (exclusion of atopy was performed by skin prick testing for common allergens¹⁷). The study was approved by the hospital ethical committee of the University Medical Center, and all patients and healthy controls gave their written informed consent.

In addition, 7 individuals were included in the bronchoscopic study who were heterogeneous with regard to diagnosis (5 patients with asthma and 2 with allergic rhinitis) and underwent bronchoalveolar lavage (BAL) 48 hours after intrasegmental bronchial challenge with allergen (segmental bronchial provocation [SBP]) as previously described.¹⁸ Airway eosinophils were obtained from BAL fluid 48 hours after segmental bronchoprovocation of atopic human subjects with relevant antigen. Antigen dose for segmental bronchoprovocation was defined as previously described (for details, see the Online Repository at www.mosby.com/jaci).^{19,20} Bronchoscopic procedures were approved by the University of Wisconsin-Madison Health Sciences Human Subjects Committee, and informed consent was obtained from all subjects before participation.

Blood sampling

Blood was obtained from healthy donors without allergy (from the laboratory staff) and patients with allergic asthma. Furthermore, blood samples were obtained from atopic subjects who underwent a segmental allergen challenge, and in a subgroup of patients with asthma, blood samples were obtained before and at 3 time points after an inhaled allergen challenge or sham challenge (using inhaled saline as control, at 3, 6, and 24 hours after the challenge, respectively).

TABLE I. Characteristics of patients with asthma (n = 38) from whom whole blood was collected (A) and from the subpopulation of this group (n = 22) who underwent an inhaled allergen challenge with a late asthmatic response (B)

	Controls Mean (SD)	A Mean (SD)	B Mean (SD)
Age, y	31 (5.6)	22 (5.6)	22 (5.5)
Gender (M/F)	22/16	26/12	16/6
Atopy	—	+	+
Baseline FEV ₁ (% predicted)	101 (2.5)	94 (10)	91 (10)
Methacholine PC ₂₀ *	—	—	0.52 (0.07-4.43)
Late asthmatic response (% fall in FEV ₁)	—	—	30(10)
Allergen†	—	—	17 House dust mite 2 Cat 3 Grass

*Geometric mean (range).

†Inhaled allergen during challenge.

Procedure for staining eosinophils with monoclonal phage antibodies A17 and A27

Priming *in vitro*. Blood was collected in tubes containing sodium heparin as anticoagulant and incubated at 37°C immediately after venapuncture. Blood was treated with buffer (control) or with different amounts of cytokines (GM-CSF and IL-5 incubated for 30 minutes at 37°C) and for different periods (concentration 0.1 nmol/L for 30 minutes) as indicated in the different figures. Hereafter, the blood was chilled to 4°C, erythrocytes were lysed, and the leukocytes were stained with antibodies.

Priming *in vivo*. Unstimulated and N-formyl-methionyl-leucyl-phenylalanine (FMLP)-stimulated (1 μ mol/L, 10 minutes at 37°C) blood samples from normal donors and patients with asthma were immediately put on ice until further processing.

The used reagents are described in the Online Repository (www.mosby.com/jaci).

Flow-cytometric evaluation of eosinophil labeling by monoclonal phage antibodies A17 and A27. Blood samples were stained with fluorescein isothiocyanate (FITC) directly labeled phage antibodies A17 and A27.¹⁵ In short, monoclonal phage antibodies (MoPhabs) A17 and A27 were diluted 1:10 with PBS:4% milk powder. This mix at 100 μ L was added to whole blood samples of 50 μ L each and incubated for 60 minutes on ice. Hereafter, the red cells were lysed in ice-cold isotonic NH₄Cl and centrifuged at 1500 rpm for 7 minutes. Pelleted cells were washed and resuspended in ice-cold PBS/1% human serum albumin for analysis.¹⁵ Cells were analyzed in a FACS vantage flow cytometer (Becton & Dickinson, Mountain View, Calif). Eosinophils were identified according their specific side scatter and forward scatter signals.²¹ Data from individual experiments are reported as fluorescence intensity in arbitrary units (AU) or summarized as the median channel fluorescence (MCF) of at least 5000 events.

Analysis of airway eosinophils. Data on airway eosinophils were acquired by using the flow-cytometric analysis of eosinophils from BAL fluid obtained 48 hours after SBP.¹⁷ The unprocessed BAL fluid containing resident airway cells was incubated with FITC-labeled A17 or A27 phage antibody, and the eosinophils were identified on the basis of scatter characteristics.

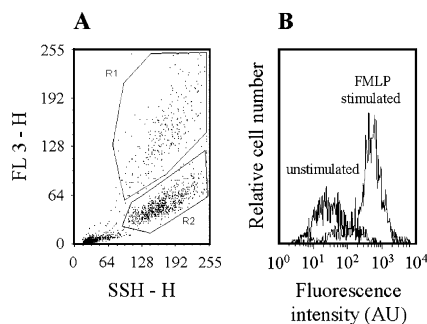


FIG 1. MoPhabs A17 and A27 recognize eosinophils activated in whole blood as described in materials and methods. **A**, Eosinophils (gate R1) and neutrophils (gate R2) were identified according to their depolarizing sideward scatter as previously described.²¹ **B**, Histograms for unstimulated and FMLP-stimulated blood eosinophils are shown for A17 blood labeling by flow cytometry.

Statistical analysis

The results are generally expressed as means $\pm$ SEMs. Statistical analysis was performed by using paired and unpaired Student *t* tests when comparing 2 groups. Otherwise, analysis of patient data and comparison with controls were performed by variance analysis using ANOVA (corrected according to Bonferroni). The Pearson correlation test was performed when indicated. All statistical tests were performed by using the statistical software package (SPSS Inc version 10.0; Chicago, Ill). In all experiments, *P* values $< .05$ were considered statistically significant.

RESULTS

MoPhabs A17 and A27 recognize primed eosinophils in peripheral blood

The identification of eosinophils in total leukocyte suspensions was performed (population in gate R1 in Fig 1, A; see reference²¹). When whole blood is stimulated for 10 minutes with 0 μ mol/L or 1 μ mol/L FMLP, the labeling of eosinophils with MoPhab A17 is markedly enhanced (Fig 1, B). Similar histograms were acquired for labeling with MoPhab A27 (results not shown). Furthermore, *in vitro* stimulation of eosinophils with single cytokines such as GM-CSF and IL-5 induce a readily detectable expression of the priming epitopes recognized by MoPhabs A17 and A27. Maximal expression was found at concentrations between 100 pmol/L and 1 nmol/L (Fig 2, A and B) and after 30 to 60 minutes of cytokine priming (Fig 3, A and B). This is in agreement with the priming of functional responses induced by these cytokines *in vitro* (for FMLP stimulation data, see Fig E1, A and B, in the Journal's Online Repository at www.mosby.com/jaci).²² The data in Figs 1 to 3 demonstrate that MoPhabs A17 and A27 are able to distinguish cytokine primed cells from normal resting cells present in peripheral blood.

Interestingly, in contrast with neutrophils, eosinophils from allergic asthmatic patients exhibit a higher expression of epitopes recognized by MoPhab A17 (Fig 4, A; *P* = .001) and A27 (Fig 4, B; *P* < .0001) than cells from

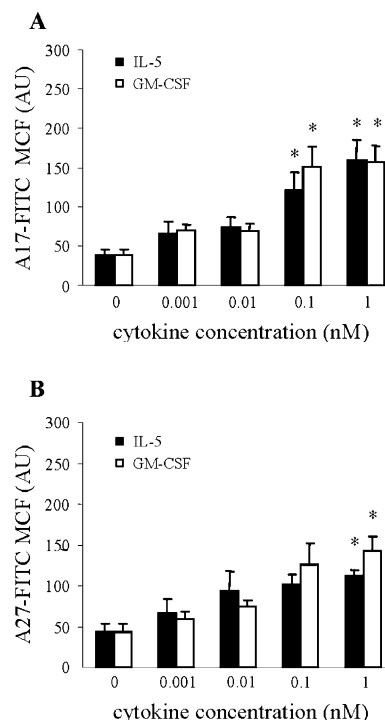


FIG 2. Dose-response curves of cytokine induced expression of priming epitopes by FITC-labeled MoPhabs (**A**, MoPhab A17; **B**, MoPhab A27) on human eosinophils in the blood of IL-5 or GM-CSF (see Methods; **P* < .01; *N* = 4.), designated by the MCF.

patients without allergy. These data suggest that eosinophils from patients with allergic asthma have a primed phenotype *in vivo*.

All of the blood samples in this study were also stimulated via the addition of a high concentration (1 μ mol/L) of the chemotaxin FMLP. As can be seen from Fig 4, C and D, activation of whole blood of both control subjects without allergy and patients with allergic asthma with FMLP induces a marked and similar increase in expression of epitopes recognized by MoPhabs A17 and A27. These findings are consistent with previous studies suggesting that partially primed cells are found in the circulation of patients with allergic asthma.

Eosinophil priming in peripheral blood increases after an allergen-induced late-phase response

The kinetics of A17 and A27 epitope expression was measured on eosinophils and neutrophils in peripheral blood after allergen challenge. We found a significant increase in priming (visualized by A17) of eosinophils, but not for neutrophils, 6 hours after allergen challenge (*P* < .01), whereas labeling by MoPhab A27 was not increased at this time point (Fig 5). This was allergen-specific, because sham challenges did not affect peripheral leukocytes (Fig 5). In addition, this eosinophil priming *in vitro* after cytokine stimulation stays enhanced for 24 hours (data not shown).

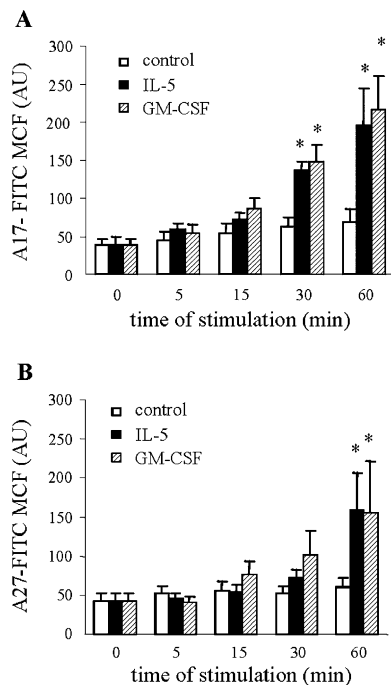


FIG 3. Time course of cytokine-induced expression of priming epitopes on human eosinophils by FITC-labeled MoPhab A17 (**A**, * $P < .01$, IL-5 and GM-CSF; $n = 5$) or MoPhab A27 (**B**, * $P < .05$ for IL-5 and $P < .01$ for GM-CSF; $n = 5$) as the MCF.

Elevated eosinophil priming in BAL fluid after segmental bronchoprovocation

An enhanced expression of the priming epitopes recognized by MoPhabs A17 and A27 was seen on blood eosinophils after allergen (48 hours) compared with cells of nonchallenged donors without allergy acquired at the same time (data not shown). Epitope A17 expression on airway eosinophils was markedly enhanced but variable from patient to patient (for patient characteristics, see Table II). The epitope recognized by epitope A27 was more abundant on the airway eosinophils than on blood eosinophils in all 7 patients (blood mean, 773; BAL mean, 2302; $P = .03$). Data are plotted for eosinophils staining by A17 (Fig 6, C) and A27 (Fig 6, D), with each line representing the slope of the difference in labeling between blood and BAL eosinophils of a separate patient. The labeling of BAL eosinophils by A27 was significantly greater than the labeling of blood eosinophils from the same donors ($P = .02$). Labeling of eosinophils in each sample was summarized as the MCF for at least 2000 events.

The difference in expression of A17 between peripheral blood and BAL eosinophils in individual patients negatively correlated with the hyperresponsiveness in these subjects ($r = -0.78$; $P < .05$). A similar trend was found with A27, but this did not reach statistical significance ($r = -0.44$; $P = .32$). Patients with a greater difference between BAL minus blood expression of the epitope for MoPhab A17 also have a lower PC₂₀ methacholine, indicating a relationship between this parameter (bronchial

hyperreactivity) and epitope expression on airway eosinophils.

DISCUSSION

The inflammatory process in the airways of patients with allergic asthma is associated with an eosinophilia in the peripheral blood and its progenitors in the bone marrow.^{22,23} However, it is not unequivocally known what cytokines or chemokines are responsible for this bone marrow response.²⁴⁻²⁶

Several studies have demonstrated that priming is essential for several eosinophil responses isolated from the peripheral blood of patients with allergic asthma, and it is not the mere quantity of cells in the peripheral blood that is important.^{11,12,27,28} Furthermore, many processes including adhesion, degranulation, and respiratory burst activated by physiological stimuli are optimally activated only provided that the cells have interacted with a priming agent. The most convincing data were acquired by measuring migratory-associated processes. Adhesion to surfaces, transendothelial migration, and chemotaxis were found to be enhanced in eosinophils isolated from the blood of patients with allergic asthma.^{12,14,27,28} However, cytotoxic responses such as degranulation and activation of the respiratory burst were less sensitive for this *in vivo* priming process.²⁸ These findings are consistent with the hypothesis that early priming processes are focused on adhesion processes and prepare eosinophils to leave the peripheral blood to inflammatory loci. Cytotoxic responses are then primed during transition from peripheral blood to the inflammatory loci. Indeed, eosinophils that have migrated through endothelium *in vitro* or cells obtained after segmental allergen challenge *in vivo* exhibit a primed phenotype in the context of cytotoxic responsiveness.²⁹

To investigate the allergen-induced response of inflammatory cells in the blood before their extravasation, we developed a method to detect the cytokine-induced priming response recognized by MoPhabs A17 and A27.¹⁵ Until now, priming could be studied only in the context of these complex functional responses, making these experiments laborious to perform. Apart from these practical difficulties, an important additional drawback is the fact that these functional assays have to be performed with isolated cells. Isolation of cells can introduce aspecific activation, which has also been evident for human neutrophils.³⁰ In addition, isolation of eosinophils takes several hours, which can preclude the study of fast and transient priming responses. Application of our antibodies recognizing priming epitopes on granulocytes and monocytes circumvents many of these problems; therefore, this method may provide an easy way to investigate the kinetics of allergen-induced inflammatory responses. With these sequential measurements, we could now show that maximal priming of eosinophils in peripheral blood occurs just before initiation of the late asthmatic response (Fig 5).

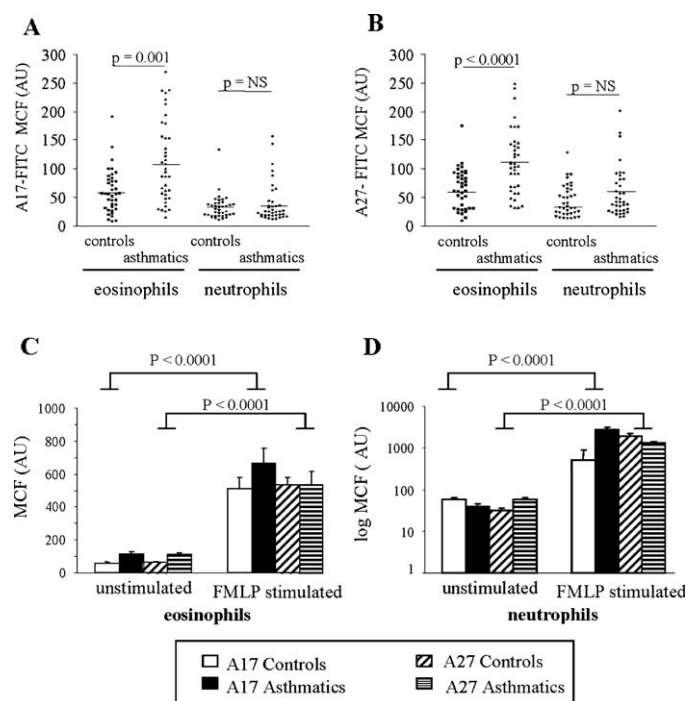


FIG 4. Expression of priming epitopes, recognized by FITC-labeled MoPhabs A17 and A27 (MCF, mean $\pm$ SEM for labeling) on eosinophils and neutrophils from blood donors with and without asthma presented by individual dot plots (**A**, A17; and **B**, A27). After FMLP stimulation, MoPhab A17 and A27 showed greater labeling of eosinophils (**C**) and neutrophils (**D**).

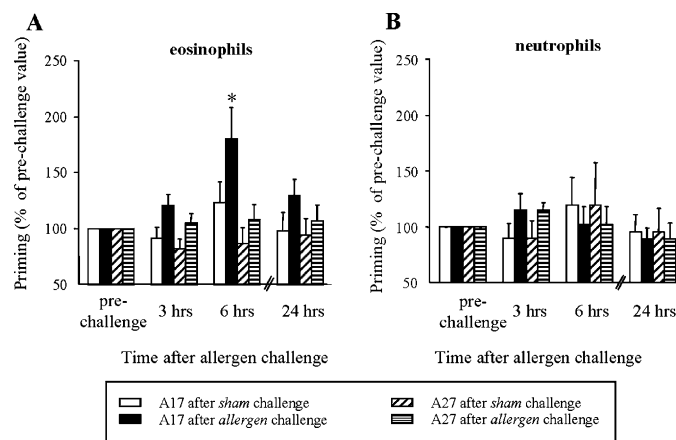


FIG 5. Kinetics of expression of priming epitopes on eosinophils in peripheral blood in response to allergen challenge in patients with asthma. The percentage change from baseline at 3, 6, and 24 hours after sham (control) or inhaled allergen challenge is expressed by MoPhab A17 and MoPhab A27 (prechallenge value set at 100%; MoPhab A17: $*P < .01$ at 6 hours).

Both A17 and A27 phage antibodies recognize cytokine-primed eosinophils in total leukocyte preparations comparable with the situation with monocytes and neutrophils (Figs 1 and 2).¹⁵ As has also been shown for adhesion-associated responses, the expression of these priming epitopes is enhanced on eosinophils isolated from the peripheral blood, again showing priming of eosinophils *in vivo*. Application of our antibodies not only facilitates greatly the study of priming *in vivo* but also

considerably extends our knowledge concerning the process of priming. First, this flow cytometry approach allows analysis of the priming response of the total phagocyte population present in the leukocyte fraction. Fig 1, B, shows that the vast majority of eosinophils are sensitive for priming in the peripheral blood, ruling out the possibility that maximal priming occurs in only a subpopulation of cells. Indeed, the expression of priming epitopes is higher when cells are activated with high concentrations of

TABLE II. Characteristics of patients with allergy who underwent a SBP and BAL

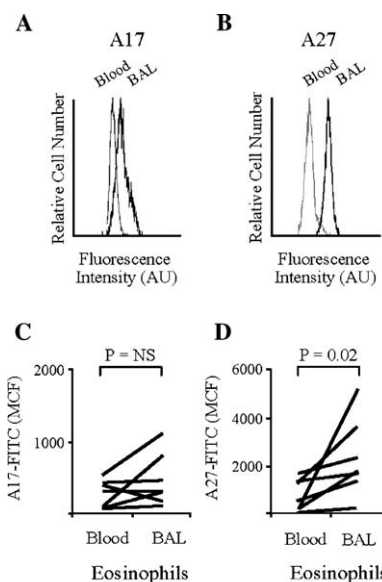
Subject	Sex	Age (y)	Baseline FEV ₁ (% predicted) before antigen challenge	Methacholine PC ₂₀ , mg/mL	Antigen
1	F	46	109	3.7	HDM
2	M	28	107	2.3	RW
3	F	20	100	0.7	Cat
4	F	24	100	9.9	RW
5	F	19	79	2.9	HDM
6	F	31	81	17.9	Cat
7	M	40	82	20	RW

HDM, House dust mite; RW, ragweed.

chemokines/chemotaxins compared with cytokines. Second, eosinophils in the peripheral blood of patients with allergic asthma are only partially primed. After addition of a potent activator to whole blood *in vitro*, the expression of the priming epitopes could be still enhanced (Fig 4, C). This finding can be explained by at least 2 hypotheses of *in vivo* priming: (1) the cytokine burden in the peripheral blood is very low and insufficient to promote high levels of expression of the MoPhabs A17 and A27 priming epitopes, or (2) the expression of high levels of MoPhabs A17 and A27 is conducive to extravasation.

To test the aforementioned hypotheses, we measured the kinetics of expression of priming epitopes on eosinophils in peripheral blood after (inhaled) allergen challenge. We found only a short burst of priming with respect to the A17 MoPhab 6 hours after inhaled allergen challenge. Eosinophil priming returned to prechallenge levels within 24 hours. The cells were still able to express the priming epitopes given that activation of these cells induced an expression of epitopes comparable with cells obtained from normal controls. These data show that inflammatory mediators in the lung induced by allergen challenge can affect systemic inflammatory cells in the peripheral blood, which previous studies also demonstrated by increased blood activation markers like CD69 and eosinophilic cationic protein after allergen challenge, despite the absence of increased adhesion molecules.^{20,31,32} The data obtained after segmental allergen challenge are in agreement with the hypothesis that highly primed eosinophils extravasate and leave the peripheral blood, which also provides an explanation of why expression 24 hours after allergen challenge returned to prechallenge conditions.

Although the current data suggest differences between the priming response of eosinophils from the peripheral blood and the pulmonary tissue, the antigens recognized by the phage antibodies A17 and A27 have not been identified. Priming of leukocytes *in vitro* by different chemokines and cytokines leads to similar increases in expression of epitopes recognized by A17 and A27.¹⁵ Both antibodies can cross-block binding (results not shown), suggesting that the epitopes either are present in the same antigen or are in close proximity (such as in a cell

**FIG 6.** Expression of epitopes for labeled FITC MoPhabs on blood and BAL fluid eosinophils 48 hours after segmental bronchoprovocation with antigen (MCF). **A and B**, individual A17 and A27 expression, respectively. **C and D**, Difference in MCF between blood and BAL eosinophils of individual patients using A17-FITC or A27-FITC, respectively (A27: $P = .02$; $N = 7$).

surface protein complex). Interestingly, activation *in vivo* clearly shows a difference in expression of these epitopes. A17⁺ eosinophils are induced in the peripheral blood after allergen, whereas A27 only becomes positive on extravasation (Figs 5 and 6). This finding is consistent with the hypothesis that activation processes *in vivo* are more complex than those induced by cytokines *in vitro*. Apparently, the epitope recognized by antibody A27 needs tissue-associated signals to become expressed.

The epitope recognized by the A17 antibody is rapidly induced on peripheral blood primed by cytokines *in vitro* or on cells primed *in vivo* in patients with allergy. Surprisingly, the expression of A17 on BAL eosinophils is variable, sometimes even lower than on cells found in the peripheral blood. However, the correlation found between the methacholine hyperresponsiveness and the differential expression of the MoPhab A17 epitope in blood and BAL eosinophils (Fig 6, B) is consistent with a model that acquisition of additional priming during extravasation is associated with more severe disease (high airway hyperresponsiveness/low PC₂₀), whereas lowering the priming response is associated with mild disease (low airway hyperresponsiveness/high PC₂₀). Expression of the epitope recognized by MoPhab A27 is markedly higher in cells obtained from the tissue and seems less sensitive for priming in the peripheral blood (Fig 6, A).

Our studies have demonstrated that MoPhabs A17 and A27 can measure eosinophil priming in the peripheral blood of patients with allergic asthma. These antibodies allow detailed studies of the priming process of phagocytes both in peripheral blood and in tissues. Eosinophil priming in peripheral blood occurs in all cells in a small

time frame and has very dynamic characteristics. These data are consistent with the hypothesis that priming of eosinophils precedes extravasation of the cells into inflammatory loci.

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