

# In vivo expression of CD69 on lung eosinophils in eosinophilic pneumonia: CD69 as a possible activation marker for eosinophils

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*The antigen, CD69, has been demonstrated to be expressed on activated T cells and natural killer cells. There have been no studies concerning the expression of CD69 on eosinophils. In this article, we demonstrate that lung eosinophils obtained from the bronchoalveolar lavage fluid of patients with eosinophilic pneumonia expressed significant levels of CD69, whereas peripheral blood (PB) eosinophils did not express CD69. We also activated PB eosinophils in vitro using phorbol myristate acetate and cytokines to determine whether CD69 was expressed. PB eosinophils expressed CD69 after short-term culture with phorbol myristate acetate and eosinophil hemopoietic cytokines (interleukin-3, granulocyte-macrophage-colony-stimulating factor, and interleukin-5). These findings suggest that CD69 may be a useful marker for activated eosinophils at inflammatory sites. (J ALLERGY CLIN IMMUNOL 1992;90:169-74)*

**Key words:** CD69, lung eosinophils, eosinophilic pneumonia, activated eosinophils, PMA, IL-3, IL-5, GM-CSF

It has been demonstrated that the antigen, CD69 (formerly called EA-1, MLR-3, Leu-23, and AIM), is expressed on activated T cells<sup>1-4</sup> and natural killer cells.<sup>5</sup> In vivo, CD69 is reported to be constitutively expressed and phosphorylated on CD3<sup>bright</sup> thymocytes.<sup>6</sup> A small number of circulating large T cells and natural killer cells have also been found to be CD69<sup>+</sup>, possibly as a result of in vivo activation.<sup>5</sup> A recent study has demonstrated that CD69 is also constitutively expressed on resting platelets and mediates activation and aggregation.<sup>7</sup> There have been no studies of CD69 expression on eosinophils. Eosinophils are associated with allergic, parasitic, and chronic inflammatory responses. There have previously been several studies describing the state of activation of PB eosinophils in eosinophilic patients. For example, PB eosinophils obtained from eosinophilic patients ex-

## Abbreviations used

|         |                                                  |
|---------|--------------------------------------------------|
| PB:     | Peripheral blood                                 |
| BALF:   | Bronchoalveolar lavage fluid                     |
| FITC:   | Fluorescein isothiocyanate                       |
| PMA:    | Phorbol myristate acetate                        |
| PBS:    | Phosphate-buffered saline                        |
| FCS:    | Fetal calf serum                                 |
| GM-CSF: | Granulocyte-macrophage-colony-stimulating factor |
| G-CSF:  | Granulocyte-CSF                                  |
| IL:     | Interleukin                                      |
| FcR:    | Fc receptor                                      |

hibit a lower density,<sup>8-11</sup> increased leukotriene C<sub>4</sub> production,<sup>12</sup> and higher levels of surface-antigen expression<sup>13</sup> when these eosinophils are compared with those from normal individuals. However, very few studies have been undertaken to demonstrate the state of activation of "local" eosinophils at inflammatory sites, and no definitive parameter for detecting activated eosinophils at inflammatory sites has been reported to date. In the present study, we demonstrate that lung eosinophils obtained from the BALF of patients with eosinophilic pneumonia expressed significant amounts of CD69, whereas PB eosinophils did not express CD69.

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**TABLE I.** Characteristics of the patients with eosinophilic pneumonia

| Case | Sex | Age (yr) | PB             |         | BALF    |           |
|------|-----|----------|----------------|---------|---------|-----------|
|      |     |          | WBC ( $\mu$ l) | Eos (%) | Eos (%) | Neutr (%) |
| 1    | M   | 62       | 11,600         | 14      | 69      | 2         |
| 2    | F   | 39       | 5,700          | 32      | 11      | 1         |
| 3    | M   | 22       | 16,200         | 47      | 57      | 1         |
| 4    | F   | 36       | 8,600          | 36      | 20      | 2         |

WBC, White blood cells; *Eos*, eosinophils; *Neutr*, neutrophil.

## MATERIAL AND METHODS

### Subjects

Four patients with eosinophilic pneumonia were examined in this study. Eosinophilic pneumonia is characterized by a pulmonary infiltration of eosinophils.<sup>14</sup> The characteristics of the patients are summarized in Table I. All the patients demonstrated eosinophilia and an increased percentage of eosinophils in BALF cells.

### Cell preparation

Lung eosinophils were obtained with BAL performed in a standard fashion as described elsewhere.<sup>15</sup> Briefly, BAL was performed with 150 ml of sterile saline in three aliquots of 50 ml with immediate vacuum aspiration after each aliquot in areas of lung parenchyma demonstrating the most marked infiltrative shadows on chest x-ray. Immediately after BAL, the fluid was filtered through several layers of surgical gauze. The cells were separated from the BALF by centrifugation (800 g for 10 minutes) and resuspended in  $\text{Ca}^{++}$ - and  $\text{Mg}^{++}$ -free PBS containing 2% FCS. The cells were then evaluated for total number and stained with May-Grünwald-Giemsa for differential cell count. At least 1000 leukocytes were identified on each smear as alveolar macrophages, lymphocytes, eosinophils, or neutrophils. For immunofluorescence staining, contaminated red blood cells in the BALF cells were lysed with  $\text{NH}_4\text{Cl}$  solution. Then, these BALF cells were subsequently washed twice with  $\text{Ca}^{++}$ - and  $\text{Mg}^{++}$ -free PBS containing 2% FCS.

PB eosinophils were obtained from heparinized venous blood by Ficoll-Paque-gradient centrifugation. Eosinophils coprecipitated with neutrophils in the high-density fraction (granulocyte fraction) with this method. Contaminated red blood cells in the granulocyte fraction were lysed with  $\text{NH}_4\text{Cl}$  solution. The granulocytes were then washed twice with  $\text{Ca}^{++}$ - and  $\text{Mg}^{++}$ -free PBS containing 2% FCS.

### Immunofluorescence and flow cytometry

Monoclonal antibodies used in this study were FITC-conjugated anti-Leu-11 (CD16) and anti-Leu-23 (CD69), purchased from Becton Dickinson Immunocytometry Systems, San Jose, Calif. FITC-conjugated isotype-matched control antibodies were used as controls to exclude non-specific binding. Immunofluorescence and flow cytometry were performed as previously described.<sup>16</sup> Cells were analyzed on a FACScan (Becton Dickinson).

### Stimulation of eosinophils with PMA and cytokines

Purified eosinophils (>90% pure), obtained from the PB of five eosinophilic patients by discontinuous density-gradient centrifugation with 22% to 25% metrizamide in Tyrode's gel/deoxyribonuclease<sup>17</sup> were cultured for 6 hours with or without PMA and various cytokines in RPMI 1640 supplemented with 10% FCS and antibiotics. Cytokines used in this study were human recombinant IL-2, IL-3, IL-5, IL-6, G-CSF, and GM-CSF. After the culture, the cells were stained with FITC-conjugated anti-Leu-23 (CD69), and the expression of CD69 was measured on a FACScan.

## RESULTS

Fluorescence-activated cell sorter profiles of a representative case (case 3) are depicted in Fig. 1. Lung eosinophils (Fig. 1, A) expressed significant amounts of CD69, whereas PB eosinophils (Fig. 1, B) did not express CD69. Similarly, PB neutrophils (Fig. 1, C) did not express CD69, whereas CD16 (Fc $\gamma$ R III) was expressed in great amounts. Identical high expression of CD69 on lung eosinophils was observed in all four cases examined in this study (Fig. 2).

Eosinophil populations can be divided into two groups according to their densities; hypodense and normodense eosinophils.<sup>8</sup> Since unseparated PB eosinophils did not express CD69, we determined whether the level of CD69 expression differ on hypodense eosinophils and on normodense eosinophils in the PB of three eosinophilic patients. In these cases, neither hypodense eosinophils nor normodense eosinophils expressed CD69, as demonstrated in a representative case depicted in Fig. 3.

We next examined the effects of PMA and cytokines on CD69 expression on PB eosinophils (Fig. 4). PMA was found to induce the expression of CD69 on eosinophils. IL-3 and GM-CSF also induced the strong expression of CD69 on eosinophils, whereas IL-5 induced small but significant levels of CD69 expression. In contrast, IL-2, IL-6, and G-CSF did not induce the expression of this antigen. Similar results were obtained in all five experiments performed in this study.

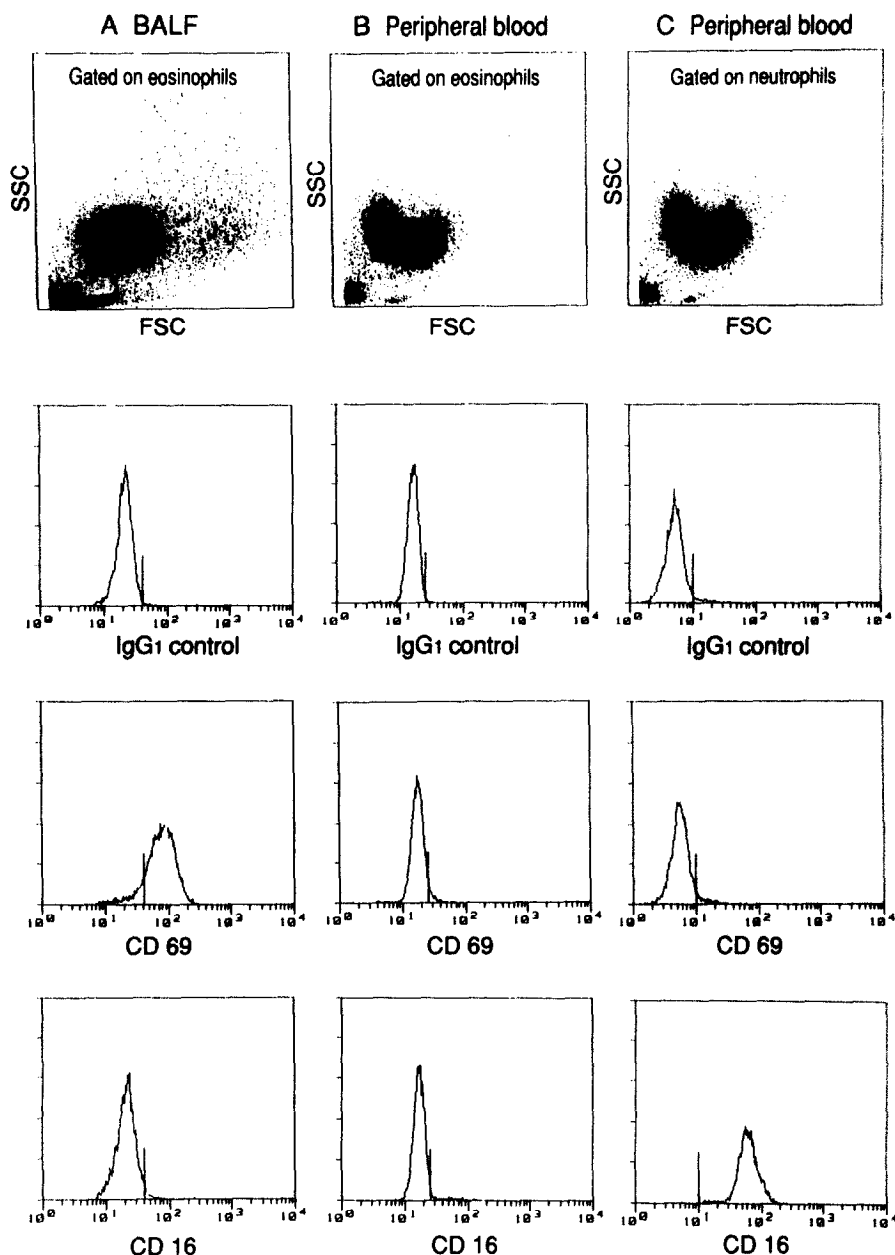


**FIG. 1.** Expression of CD69 on BALF (case 3). **A**, Lung eosinophils expressed significant amounts. **B**, PB eosinophils did not express any amount. **C**, PB neutrophils did not express any amount.

## DISCUSSION

Previous studies have demonstrated that hypodense eosinophils in PB from eosinophilic patients exhibit higher levels of  $\text{Fc}\gamma\text{R}$ ,  $\text{FC}\epsilon\text{R}$ , and complement-receptor expression, higher levels of cationic protein exocytosis, and elevated in vitro leukotriene production when these hypodense eosinophils are compared with normodense eosinophils.<sup>9, 18-21</sup> These "hypodense" eosinophils are thus considered to be in an "activated" state. However, as demonstrated in our study, neither

hypodense eosinophils nor normodense eosinophils obtained from PB expressed CD69. In contrast, CD69 was expressed strongly on lung eosinophils (tissue eosinophils). In this respect, CD69 expression on eosinophils is different from that of previously described "activation markers" of eosinophils.

The CD69 antigen is a disulfide-linked homodimer that is induced very early during lymphoid activation.<sup>1-5</sup> Its expression, which requires the synthesis of new RNA and protein, is strictly dependent on protein

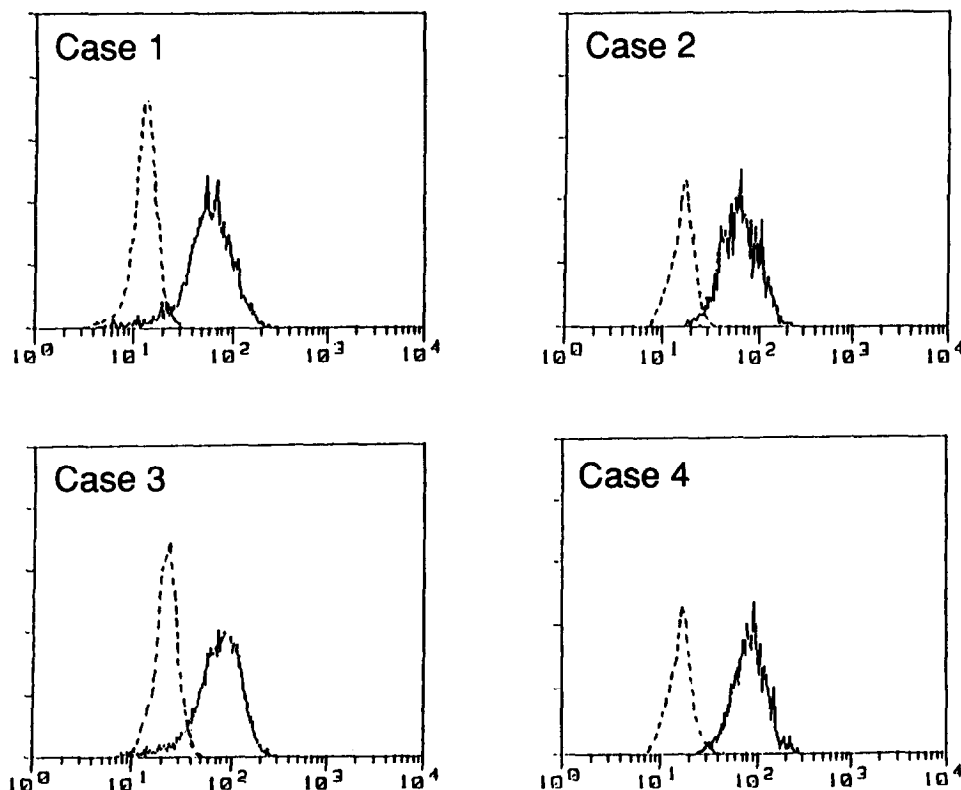


FIG. 2. Expression of CD69 on BALF eosinophils in four patients with eosinophilic pneumonia.

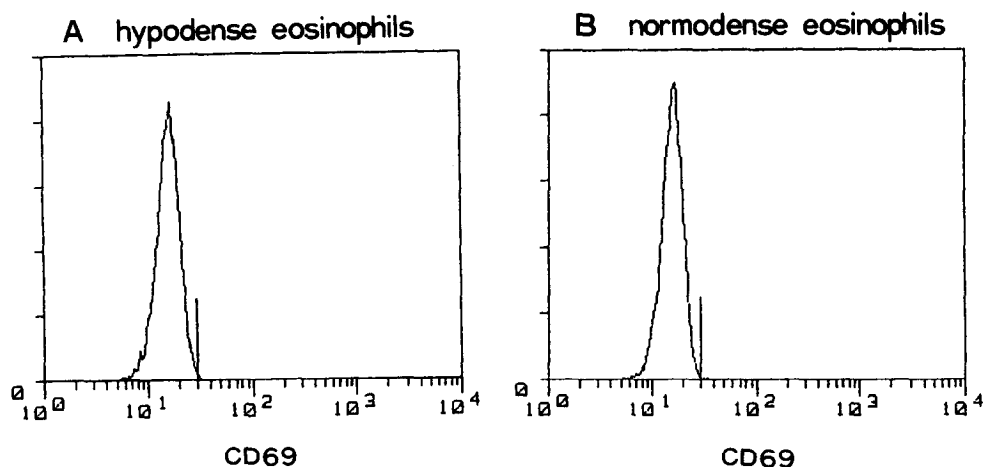


FIG. 3. Comparison of CD69 expression on A, hypodense eosinophils and B, normodense eosinophils.

kinase C activation.<sup>4</sup> To determine whether PB eosinophils express CD69 after activation, we activated PB eosinophils in vitro by short-term culture with PMA, a direct protein kinase C activator. As illustrated in Fig. 4, PMA-stimulated eosinophils expressed significant amounts of CD69. PMA is an effective activator of eosinophils but is far from a physiologic

substance. Thus, we next tested "eosinophil hemopoietic factors," such as IL-3, GM-CSF, and IL-5 as activators. IL-3, GM-CSF, and IL-5, all induced significant CD69 expression on PB eosinophils after short-term culture. We also tested other cytokines, such as IL-2, IL-6, and G-CSF as controls. IL-2, IL-6, and G-CSF did not induce CD69 expression on PB

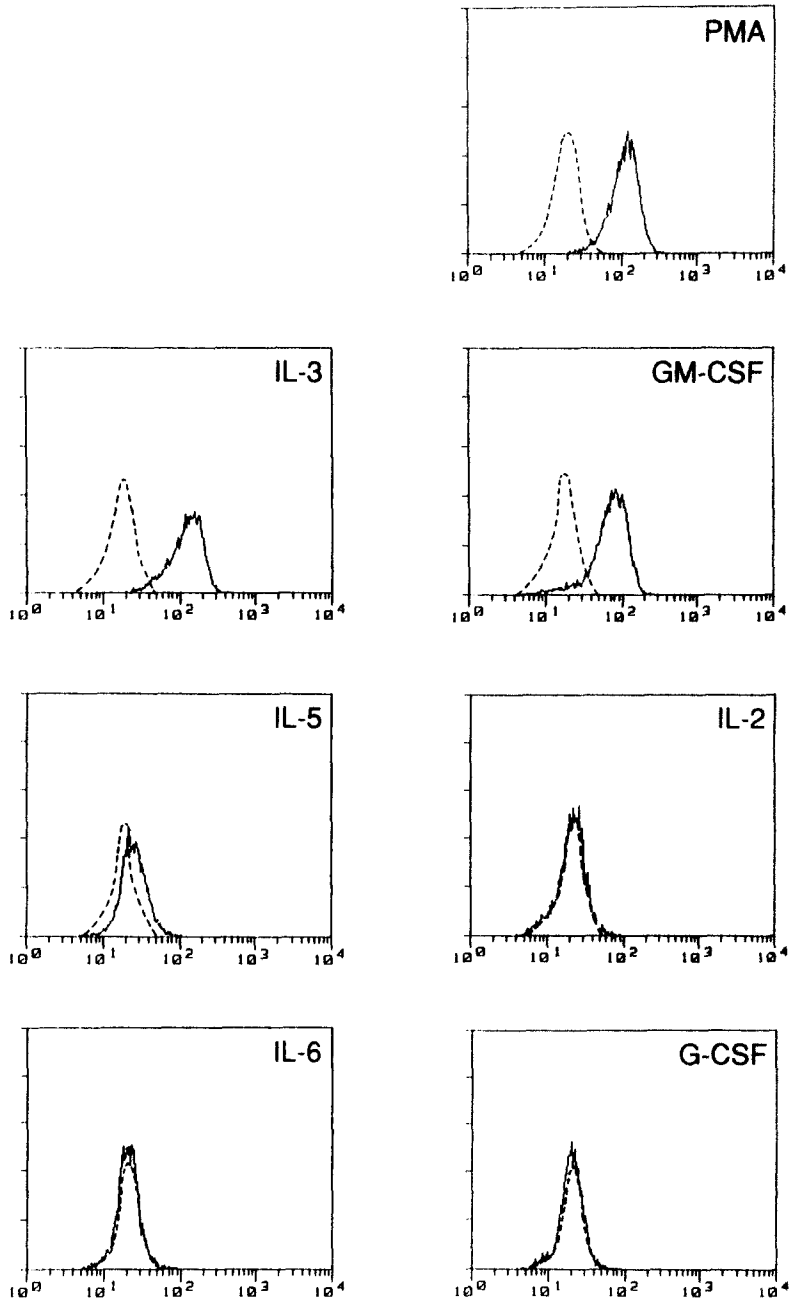


**FIG. 4.** Effects of PMA and cytokines on the induction of CD69 expression on PB eosinophils, cultured for 6 hours with PMA (2 ng/ml), IL-3 (1 ng/ml), GM-CSF (1 ng/ml), IL-5 (100 ng/ml), IL-2 (100 U/ml), IL-6 (1,000 U/ml), or G-CSF (10,000 U/ml). Expression of CD69 on eosinophils after culture with medium alone (---) or various regents (—) were analyzed on FACScan.

eosinophils. The *in vitro* induction of CD69 expression on PB eosinohils, not only by PMA but also by eosinophil hemopoietic factors (IL-3, GM-CSF, and IL-5), supports the idea that the *in vivo* expression of CD69 on lung eosinophils observed in this study may be an activation signal for eosinophils at the inflammatory site. We also found that eosinophils obtained

from pleural effusion of a different patient with hypereosinophilic syndrome, rather than eosinophilic pneumonia, expressed CD69 (manuscript in preparation), indicating that CD69 expression on eosinophils may indicate a more general activation of eosinophils.

Although we have not examined the expression of

other lymphocyte activation markers, such as HLA-DR on lung eosinophils, a recent study has demonstrated that HLA-DR expression can be induced on PB eosinophils after culture with GM-CSF.<sup>22</sup>

In conclusion, our results are of great interest not only because CD69 could provide an activation marker for eosinophils independent of density or function but also because CD69 expression, a marker previously believed to be restricted to lymphocytes, on eosinophils confirms the intriguing association of eosinophils and lymphocytes.

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