

A comparative study of eosinophil isolation by different procedures of CD16-negative depletion

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Eosinophils were isolated by the three methods of CD16-negative depletion: 1) magnetic beads, 2) fluorescence-activated cell sorter (FACS), and 3) complement reaction. Their purity, yield, and viability were compared. The second procedure produced well purity and viability ($94.65 \pm 1.51\%$ and $94.98 \pm 1.40\%$, respectively) but low yield of eosinophils ($65.47 \pm 2.47\%$). The viability of cells obtained by the third procedure was not efficient ($80.83 \pm 2.85\%$), while the purity and the yield were efficient ($96.23 \pm 1.09\%$ and $90.75 \pm 1.72\%$, respectively). In conclusion, the magnetic beads method (purity: $98.02 \pm 0.45\%$, yield: $91.05 \pm 2.43\%$, viability: $97.57 \pm 0.37\%$) was the most advantageous of these three procedures. Moreover, in the functional assay, radical oxygen products from eosinophils isolated by the procedure with complement reaction were less than with the magnetic beads or FACS procedures.

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Eosinophilia is observed in a wide variety of allergic diseases. The knowledge now accumulated suggests that eosinophilia in the peripheral blood or tissue is related to parasitic diseases and many types of allergic reaction (13). Furthermore, eosinophils are known to function as effector cells that play an important role in the pathogenesis of allergic inflammatory reactions such as bronchial asthma (1). Therefore, studies on the function and structure of eosinophils contribute to our greater understanding of the pathogenesis of allergic disorders. In such studies on eosinophils, it is necessary to obtain eosinophils at high purity with high yield. Various methods have been reported, including the discontinuous density gradient method using percoll and other polymers (2, 7, 8, 11, 12, 14, 15). However, consequently, the isolation of eosinophils at high purity or high yield is difficult. As an alternative, CD16-negative depletion, which was developed from the finding that neutrophils express CD16 when eosinophils are CD16-negative cells, has been introduced recently (10). On the basis of this report, we compared eosinophil isolation by the three different methods.

Material and methods

Three different procedures for eosinophil isolation

Isolation of eosinophils from the peripheral blood obtained from six eosinophilic patients whose leukocyte differential count showed more than 30% eosinophils in the peripheral blood was examined in the study. After addition of a 1/4 volume of 6% high-mol.-wt. dextran solution (Wako Chemicals, Osaka, Japan) to the heparinized peripheral venous blood, the mixture was allowed to stand for 40 min at room temperature, and the buffy coat was centrifuged with Ficoll-Paque (specific gravity: 1.077; Pharmacia, Uppsala, Sweden). Polymorphonuclear leukocytes (PMNs) were harvested in the bottom layer. PMNs were washed twice with Hanks' balanced salt solution (HBSS), and then the cells were reacted with anti-CD16 monoclonal antibody (MG38: Nichirei, Tokyo) for 45 min at 4°C. After the reaction, the cells were again washed twice with HBSS. The subsequent procedures varied, as follows:

1) PMNs were reacted with antimouse IgG magnetic beads (Dynabeads, Dynal, Norway) (cell:bead

ratio = 1:20) at 4°C for 45 min. Then, CD16-positive cells were removed with a magnetic particle concentrator (MPC, Dynal, Norway), and the CD16-negative cells were harvested as eosinophils.

- 2) After addition of FITC-labeled antimouse IgG (Tago, Inc, Burlingame, CA, USA), PMNs were incubated at 4°C for 45 min. Cells were then washed and subjected to forward-scatter and side-scatter analyses with a fluorescence-activated cell sorter (FACStar®, Becton-Dickinson, Mountain View, CA, USA), as previously reported (15). Cells that showed the strongest fluorescence intensity (CD16-positive cells) in a certain condition were removed. The CD16-negative cells were obtained as eosinophils.
- 3) An equal volume of baby pure rabbit complement (Cedarlane Laboratories Ltd, Ontario, Canada) dissolved in an ice-cooled condition was added to PMNs. The mixture was shaken in a water bath at 37°C for 30 min for reaction. After washing, CD16-positive cells that were lysed as a result of reaction with the complement were removed to isolate cells that were not lysed.

In each of these three procedures, the total number of cells was counted, and cyto-spin (Shandon, Inc, Pittsburgh, PA, USA) was performed. Thereafter, May-Giemsa staining was done to identify cell fractions. When eosinophils treated with anti-CD16 antibody were used in each of the three procedures, the yield of each procedure was defined as the ratio (%) of eosinophils after completion of the procedure to those before the procedure. Cell viability was determined by exclusion after staining with trypan blue dye. From these evaluations, the purity, yield, and viability of eosinophils were evaluated in the three different procedures of CD16-negative depletion.

Functional assay by luminol-dependent chemiluminescence

Radical oxidative products in terms of luminol-dependent chemiluminescence were examined. To the mixture of 50 µl eosinophils (2×10^6 /ml) and 50 µl luminol (5×10^{-4} M), 50 µl Ca^{2+} ionophore A23187 (final concentration: 10^{-5} M) was added, and radical oxygen products were determined for 600 s with TD-4000 (Labscience Co, Japan). A peak value and an integral value were determined.

Statistical analysis

Data were expressed as mean $\pm$ SE. Student's *t*-test was used for comparison of means, a *P* value of 0.05 or less being considered to be significant.

Table 1. Percentages of purity, yield, and viability of eosinophils isolated by three different procedures

	Purity	Yield	Viability
Magnetic beads	98.20 $\pm$ 0.45	91.05 $\pm$ 2.43	97.57 $\pm$ 0.37
FACS	94.65 $\pm$ 1.51	65.47 $\pm$ 2.47	94.98 $\pm$ 1.40
Complement	96.23 $\pm$ 1.09	90.75 $\pm$ 1.72	80.83 $\pm$ 2.85

Results

The purity, yield, and viability of eosinophils isolated by the procedure using magnetic beads were 98.20 $\pm$ 0.45%, 91.05 $\pm$ 2.43%, and 97.57 $\pm$ 0.37%, respectively. The respective values were 94.65 $\pm$ 1.51%, 65.47 $\pm$ 2.47%, and 94.98 $\pm$ 1.40% with cell sorter (FACS), and 96.23 $\pm$ 1.09%, 90.75 $\pm$ 1.72%, and 80.83% $\pm$ 2.85% with complement reaction (Table 1). No significant differences in purity among these three procedures were observed (*P* > 0.1; Table 1). However, the yield of eosinophils isolated by FACS was lower than those of the other two procedures (*P* < 0.001; Table 1), and the viability of cells isolated by complement reaction was less than with the other procedures (*P* < 0.01; Table 1).

The productivity of radical oxygen products determined by luminol-dependent chemiluminescence evoked with A23187 in eosinophils isolated by magnetic beads showed the peak value of 15.52 $\pm$ 3.19

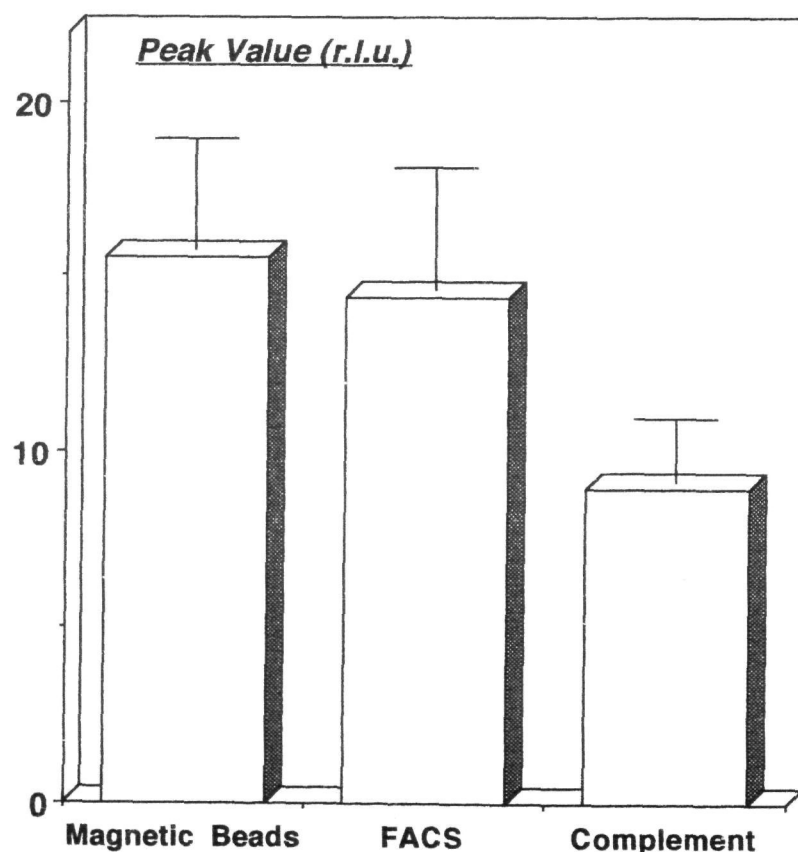


Fig. 1. Peak value of radical oxygen products measured by luminol-dependent chemiluminescence. Peak value of chemiluminescence from eosinophils isolated by complement method (8.93 $\pm$ 1.60 rlu) was lower than that of magnetic beads method (15.52 $\pm$ 3.19 rlu; *P* < 0.05) or procedure using FACS (14.38 $\pm$ 3.51 rlu; *P* < 0.05) (*n* = 3).

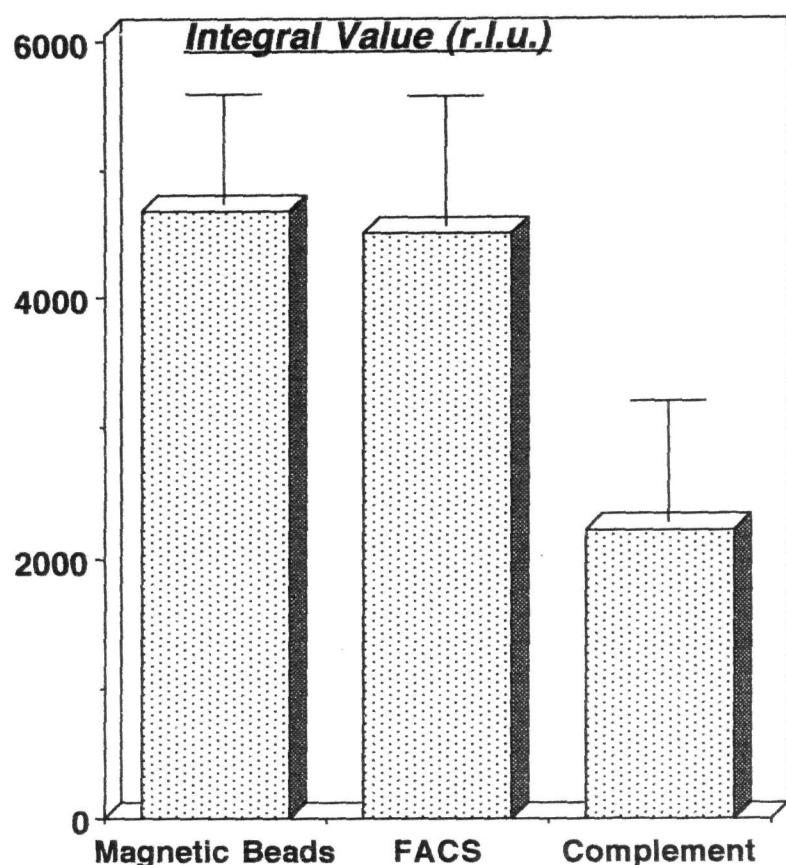


Fig. 2. Integral value of radical oxygen products measured by luminol-dependent chemiluminescence. Integral value of chemiluminescence from eosinophils isolated by complement method (2216.67 ± 929.96 rlu) was lower than that of magnetic beads method (4687.33 ± 846.50 rlu; $P < 0.05$) or procedure using FACS (4506.00 ± 1002.78 rlu; $P < 0.05$) ($n = 3$).

relative light units (rlu) and the integral value of 4687.33 ± 846.50 rlu. In eosinophils isolated by FACS, the peak value and integral value were 14.38 ± 3.51 rlu and 4506.00 ± 1002.78 rlu, respectively, whereas with the complement method, the peak value and integral value were 8.93 ± 1.60 rlu and 2216.67 ± 929.96 rlu, respectively (Figs. 1 and 2). Not only the peak value but also the integral value of chemiluminescence from eosinophils isolated by the complement method was lower than those of eosinophils isolated by magnetic beads or by FACS ($P < 0.05$; Figs. 1 and 2).

Discussion

Many recent studies on eosinophils have substantially contributed to the elucidation of the pathogenesis of allergic diseases, such as bronchial asthma, in which eosinophils are involved (1, 4, 13). Several characteristics of human eosinophil heterogeneity, as well as the existence of eosinophil subpopulations, called "normodense" and "hypodense", in diseases associated with hypereosinophilia, have been reported (3). Hypodense eosinophils can be distinguished by the increased expression of various membrane receptors, including the interleukin-5 receptor (6), and various forms of protein expression (9). The functions of eosinophils in immunologic reactions and their heterogeneity have recently become of great interest.

However, there are still some methodologic problems relating to human eosinophil isolation. The conventional modalities are that using multilayered metrizamide (14) or percoll (7), that using *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) (12), and the Nycodenz method (2). However, it cannot be ruled out that the eosinophils obtained by those methods fail to retain their native properties. In addition, some problems remain to be solved with respect to the yield and purity of eosinophils. For example, when specimens are obtained from hyper-eosinophilic patients, yield and purity are believed to be inefficient because hypodense eosinophils increase in such states of eosinophilia (5). Eosinophils can be separated from neutrophils by pretreatment with FMLP, which magnifies the difference in specific gravity between the two sorts of leukocytes (12). According to this method, however, eosinophils may be activated by FMLP (16). The metrizamide method also presents a problem because metrizamide solution is hypertonic.

In this work, we compared three different procedures of CD16-negative depletion, which were developed from the finding of a difference between CD16-negative eosinophils and CD16-positive neutrophils (10). Of the three procedures, those using FACS or rabbit complement were found to be inefficient for the following reasons. In cell sorting by FACS, there are some difficulties in selection of sorting window because of the autofluorescence of eosinophils (15). The yield of input eosinophils was reduced when we attempted to increase the purity of eosinophils. If a lower fluorescence intensity is used for cell sorting, an improvement of yield is certainly possible, but it may result in an extreme reduction in purity (data not shown). Furthermore, this procedure was rather time-consuming. On the other hand, this procedure, which does not require any special apparatus, is easy to perform, but the viability of cells obtained was low. One possible explanation of this result is that the viability of eosinophils was affected by various proteases released from lysed neutrophils as a result of complement binding during incubation. Moreover, in the functional assay, radical oxygen products from eosinophils isolated by this procedure were less than from eosinophils isolated by magnetic beads or FACS. We therefore conclude that the procedure using magnetic beads was the most advantageous method of the three, although further studies are needed to assess the function of eosinophils isolated by different methods.

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