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IgG RECEPTORS ON EOSINOPHILS : - USING IMMUNE SCANNING ELECTRON MICROSCOPY

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Abstract

Using carboxylate modified latex particles covalently conjugated with anti-IgG, IgG receptors on eosinophils were examined with scanning electron microscopy (SEM). After block and inhibition tests, significant number of latex particles were confirmed to bound on the surface of eosinophils. Earlier reports described that density of eosinophils decreased in hypereosinophilic patients and the heterogeneity of eosinophils came into focus. Our experiment revealed that eosinophils of hypereosinophilic patients had more IgG receptors than those of normal volunteers. This difference might be due to the heterogeneity of eosinophils.

Key words: Eosinophils, Hyper eosinophilic patient, IgG receptors, Latex particle, Immune scanning electron microscopy

Introduction

IgG receptors on the surface of eosinophils have been shown by the use of the rosette formation of rabbit red blood cell[7]. In recent years, the heterogeneity of eosinophils has also come into focus in the hematological[3] and immunological areas [4, 5].

In order to demonstrate and morphologically quantify IgG receptors on eosinophils, we examined the number of IgG receptors on these cells by using the carboxylate modified latex particles covalently linked to anti-IgG and counted the number of latex particles as an index of IgG receptors observed by scanning electron microscopy (SEM). We further investigated the heterogeneity of eosinophils in hypereosinophilic patients and normal volunteers by comparing the number of beads, representing IgG receptors, on the surface of eosinophils.

Material and Method

Ten cases with hypereosinophila in the peripheral blood (absolute eosinophil counts of more than 1000 per microliter) were examined. Ten cases consisted of 4 cases with pulmonary infiltration with eosinophia (PIE) syndrome and 6 cases with bronchial asthma. For control, 15 normal volunteers whose eosinophil counts in the peripheral blood were less than 200 per microliter, were also examined.

Separation of eosinophils was done by the method of Roberts[2] as shown Fig 1. Briefly, 20 ml of the peripheral blood was incubated with 10^{-6} M of formyl-Methionyl-Leucyl-Phenylalanine (f-MLP) at 37°C for 15 min. This mixture was layered 75% and 65% Percoll discontinuous gradient solution with phosphate buffered saline (PBS) and centrifugated for 25 min at 180g. Then cells between 65% and 75% Percoll solution were collected and washed by PBS for 3 times. The number of cells were counted and the percentage of eosinophils was calculated. These cells were resuspended in RPMI-1640 solution (1 x 10^6/ml) and incubated with 100 microliter of anti-human IgG (Hoechst) at 37°C for 20 min and then washed twice with RPMI.

Immunolatex was prepared by the covalently coupling carboxylate modified latex particles (0.455 micrometers in diameter) to anti-rabbit goat IgG (Hoechst) by the method of Molday[2]. One hundred microliters of this immunolatex solution were incubated with 100 microliter of eosinophil solution at 4°C for 60 min and washed twice with RPMI. Blocking control was done with saline instead of anti-

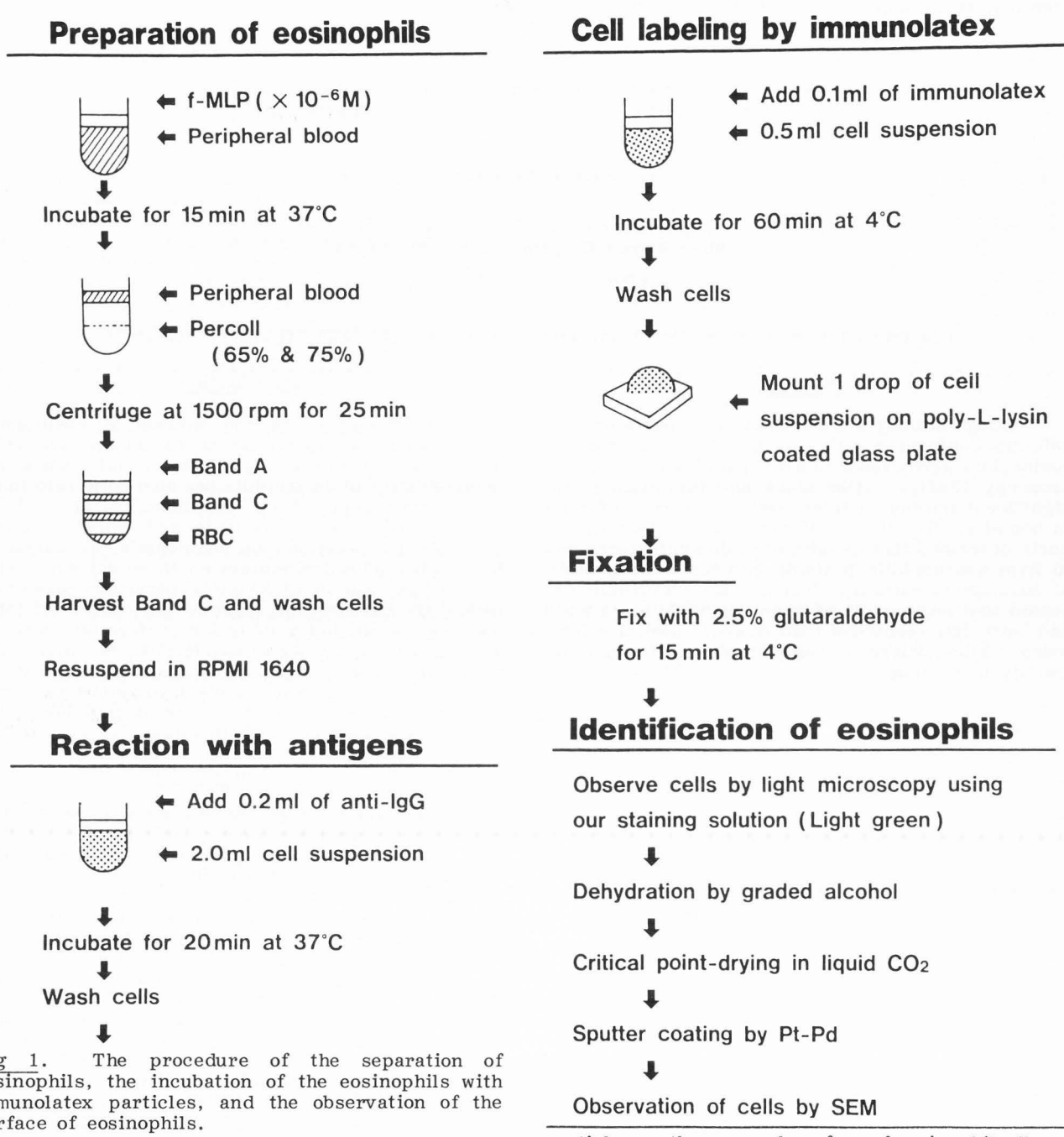


Fig 1. The procedure of the separation of eosinophils, the incubation of the eosinophils with immunolatex particles, and the observation of the surface of eosinophils.

human IgG for all cases. And the same doses of immunolatex was used. On the other hand, for 10 cases with both groups, non labeled 100 times higher concentration (0.1mg/ml) of anti-rabbit goat IgG were coincubated with latex solution for the inhibition control.

The reacted solutions were placed on poly-L-lysine coated glass for 10 minutes and then washed with cacodylate-buffered solution and fixed with 2.5% glutaraldehyde solution for 1 hour. After the dehydration with graded alcohol, critical point drying and the sputter coating with gold, eosinophils were observed by JEOL T-330 SEM. Twenty eosinophils were examined in each case and the number of latex particles on the exposed surface of eosinophils (hemisphere) was counted and used as an index of surface IgG receptor. Mean value and standard deviations were obtained for comparison Student's t-test.

Results

Purity of eosinophils in our experiment was more than 90%; and there were no differences in size between hypereosinophils and normal eosinophils. The scheme of the latex binding to eosinophils is shown Fig 2. Fig 3 shows an IgG receptor positive cell. Latex particles are bound to the surface of eosinophil. Fig 4 shows an inhibited control without immunolatex binding, indicating the specificity of the

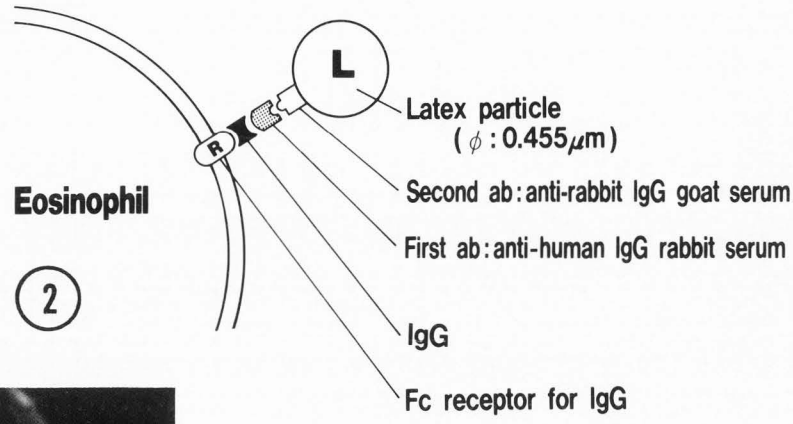
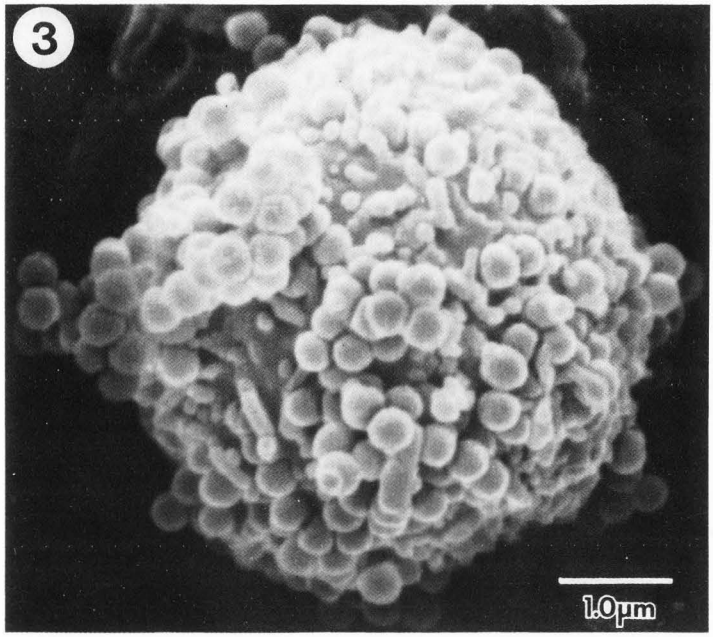
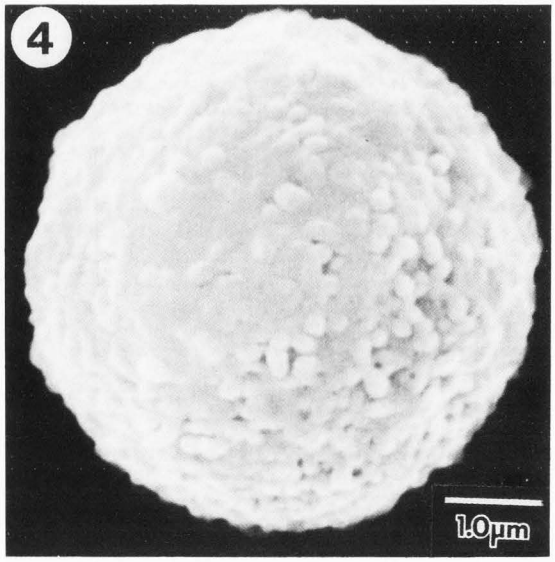


Fig 2. The scheme of the IgG receptors on the surface of eosinophils and the identical method anti-IgG conjugated latex particle.

Fig 3 (below left). Many latex particles bound on the surface of eosinophil. Fig. shows an IgG receptor positive cell.

Fig 4 (below right). An inhibited control cell. Latex bindings are not observed.





reaction. The average number of particles on the eosinophils obtained from hypereosinophilic patients was 19.0 ± 2.5 per cell. There were no differences between cases with PIE syndrome and bronchial asthma. In normal volunteers this was 7.8 ± 0.9 per cell. Corresponding figures for inhibition controls were 2.5 ± 0.6 and for blocking control were 1.0 ± 0.2 per cell. Cells from hypereosinophilic patients had significantly (p less than 0.01) more latex particles than those of normal volunteers. Furthermore, the number of latex particles on eosinophils of normal volunteers was significantly higher than that of inhibition control (p less than 0.05) and blocking control (p less than 0.01). We could not detect degranulated eosinophils in this experiment.

Discussion

The heterogeneity of eosinophils (normodense and hypodense) has been shown in various studies[1]. Although the function of the hypodense eosinophils has been considered to be different from that of normodense eosinophils, no explanation for this difference has been provided. Show et al.[6] have reported that IgG-dependent stimulation on eosinophil generated SRS-A leukotrienes. In order to examine the IgG receptors on these two kinds of eosinophils, we used the method of immune SEM (i.e., covalently anti-IgG conjugated carboxylate modified latex particles). In this method, we could semiquantitatively count the number of the latex particles on the surface of eosinophils, as representative of IgG receptors. This could not be done by other techniques, either because the heterogeneity of cells does not permit the use of such labels as ^{125}I, or because when other morphological markers (such as red blood cells) were used, they were too large for semiquantification. In this regard the smaller latex particles seem to provide an advantage.

In these experiments, the number of particles on eosinophil were significantly higher than those of inhibited and blocked controls, suggesting the specificity of the reaction. Eosinophils obtained from hypereosinophilic patients had much more IgG receptors than those of normal volunteer group. The measurement of leukotrienes generated from eosinophils of hypereosinophilic group and normal volunteer is now being done. However, we could not detect morphologically degranulated cells in our experiments. We suggest that the difference of IgG receptors on hypodense and normodense eosinophils may explain the heterogeneity of eosinophils.

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Discussion with Reviewers

J. Beesely: Is there any difference in labelling between PIE and bronchial asthma eosinophils? Could you comment whether one would or would not expect to see differences?

Author: There are no differences in labelling between PIE and bronchial asthma eosinophils.

J. Beesely: A difference in size of different eosinophils might affect the numbers of probes attaching but not necessarily the number of receptors per square micrometer. Are there any differences in sizes between "hypereosinophilic" and "normal" eosinophils? If so, will you comment on this please?

Author: There are no differences in sizes between "hypereosinophilic" and "normal" eosinophils.

J. Beesely: For hypereosinophilia, normal and inhibition control experiments n = 10, 15 and 10 respectively . Why do you use n = 25 for blocking control? Also, were these two types of control carried out normal or hypereosinophilic samples?

Author: N = 25 was used to confirm the specificity of this experiment. Controls were carried out on normal and hypereosinophilic samples.

J. Beesely: If eosinophils were degranulating would this affect the number of probes attaching?

Author: If eosinophils were degranulating, capping of probes might be observed like basophils. But I did not find degranulated eosinophils in this experiment.

Reviewer II: How was the number of Latex particles per cell determined, i.e., counting the number on the side of the cell which could be observed (half) or by rotation of the sample to visualized the whole surface area of the cell?

Author: We observed the hemisphere of eosinophils and counted the number of Latex particles. We did not use the rotation of the sample.

Reviewer III: Why is it so important to demonstrate IgG receptors on eosinophils? If indeed there are deviations in its quantities, what does this mean? Why not use radioimmunoassay? What was wrong or missing in previous studies presenting IgG on eosinophils? What type of IgG receptors did you study?

Author: The role of IgG receptors on eosinophil is still unclear and the heterogeneity of eosinophils has come into focus. We wondered about the relationship between the number of IgG receptors and their density on eosinophils; therefore we performed the experiments reported here. We believe that the size of the latex particles used was suitable for the marker of IgG receptors on eosinophil. We studied only Fc receptors (see Fig. 2).

Reviewer III: Why did you use such a "gigantic" size of latex? How did you count for cells forming aggregates after labeling? How did you know that a given labeled cell is an eosinophil and not, for example, a lymphocyte showing IgG receptor? Why were only 20 cells from each specimen examined if you have thousands of cells in each?

Author: 0.455 micrometers latex bead was sufficient enough for segregating the small organelles on the surface of eosinophil. No cell aggregation was observed. Eosinophil is larger in size than lymphocyte and other cells. We counted the number latex particles by the picture. Twenty is appropriate number to count.

Reviewer III: How do unlabeled eosinophils look under the SEM? How did they differ from other cell types encountered in your preparations (upto 10%)? How do you know that the cell in Fig. 4 is an eosinophil and not a lymphocyte (compact, villous)? How do you know for sure that labeled cell in Fig. 3 is an eosinophil and not another cell displaying IgG receptor? How can degranulated eosinophils be detected by SEM and why you could not detect them? Did you mean that degranulated cells did not exist in your preparations? In general, how many eosinophils were positively labeled, and how many particles-per-cell were regarded as positive labeling?

Author: Before preparing SEM samples, we stained cells with light green and confirmed which cells were eosinophils. And by SEM, eosinophil is larger in size than other cells, and fine microvilli on the surface are typical findings for eosinophils. In cell suspension, about 90% are eosinophils and other 10% are mainly lymphocytes. It was easy for us to segregate eosinophils from other cells by the size and shape.

Reviewer III: What are the 'hypodense and normodense' eosinophils? How can they be differentiated under the SEM? What was the rationale that immuno-SEM will by advantageous over RIA for studying these cells; do they label differently? Are 'degranulated' cells related to either one of these types? If in your results you did not mention finding two subpopulations, then why did you not use RIA alone or in parallel to your SEM studies?

Author: In our laboratory we cannot use RI, therefore we used latex beads. Eosinophils obtained from hypereosinophilic patients appeared highly hypodense; we confirmed this by the use of TEM and will publish it elsewhere. We previously found that mast cells degranulated by the capping of latex particles. We wanted to compare eosinophils with mast cells.