

Purification of Human Basophils by Affinity Chromatography on Anti-IgE-Sepharose 6MB

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This work describes a method for the purification of basophil leukocytes from human peripheral blood by the use of a three-step separation technique including affinity chromatography on anti-IgE-sepharose 6MB. The purity of the obtained basophils was 50–95 % and the recovery was 30–40 %. The basophils separated by this method appeared normal and were found to be reactive with anti-IgE in subsequent tests.

Key words: basophils; purification.

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A problem of great interest is the purification of basophils from human peripheral blood, in order to design experiments with a pure basophil system for the study of hypersensitivity phenomena of the immediate type. Some separation techniques have been described during recent years. Pruzansky & Patterson (11) obtained good purity using a two-step method including initial centrifugation on Ficoll-sodium metrizoate followed by a fractionation using glass-bead columns. Day (3) obtained very good separation using a three-step method with initial centrifugation on Ficoll-Hypaque followed by equilibrium density gradient centrifugation. Ishizaka et al. (6) have used a two-step method to obtain a basophil-rich fraction. The cells were centrifuged on a Ficoll-Hypaque layer by the method of Böyum (2) and further fractionated by chromatography on a glass-bead column as described by Rabinowitz (12). Dvorak et al. (4) have presented a method for purification of basophils from guinea pig blood and bone marrow using a Ficoll-density gradient. Affinity chromatography has not yet been used for separation of basophils though it has become useful when separating lymphocytes (5, 9, 14).

In this work we will present a three-step method for purification of basophils. First,

most of the erythrocytes are eliminated through a sedimentation procedure. In the second stage basophils and mononuclear cells are separated from the remaining erythrocytes, polymorphonuclear neutrophils and eosinophils. This stage involves centrifugation on Ficoll-Hypaque and is a modification of the initial stage in the method described by Day (3). Finally, the cells are chromatographed on an anti-IgE-sepharose column, which enables affinity fractionation of the basophils.

MATERIAL AND METHODS

Separation of basophils

Stage 1. Leukocytes were obtained from the blood of volunteers by dextran sedimentation as described by Lichtenstein & Osler (7). The leukocyte-rich supernate was removed and diluted with an equal volume of Tris A buffer, consisting of 0.025 M Tris pH 7.5, 0.120 M sodium chloride, 0.005 M potassium chloride and 0.3 mg/ml of human serum albumin. The suspended cells were used in the second stage of the separation. The first stage can be omitted if a faster separation is needed. One part anti-coagulated blood is mixed with three parts Tris A

buffer and the mixture is used in the second stage.

Stage 2. This is a modification of the first step in the method described by Day (3). The separation fluid consisted of 10.5% (w/v) sodium diatrizoate (Hypaque sodium, Winthrop Laboratories Ltd.) and 6.4% (w/v) Ficoll (Pharmacia Ltd.). The final solution had a density of 1.080 g/ml, at 20°C, which was checked with a pycnometer.

Six ml of the solution from stage 1 was carefully layered over 3 ml Hypaque-Ficoll mixture in a plastic tube. This was facilitated by the use of a 10-ml syringe barrel attached to an infusion-pump, which was set at a speed of 1 ml/min. Two or more such tubes were prepared and the tubes were centrifuged in a swing-out rotor for 30 min at 400 g at 20°C. After centrifugation, the interface, containing the basophils and mononuclear cells, was removed and centrifuged for 15 min at 270 g at 20°C. The cells were washed in Tris A buffer and finally suspended in Tris ACM buffer (Tris A-buffer containing 0.6 mM calcium and 1 mM magnesium).

The cell suspension was adjusted to approximately 1×10^{-7} cells/ml with Tris -ACM-buffer. This solution was used in the third step.

Preparation of anti-IgE-sepharose-6MB

Stage 3. Anti-IgE (Hoechst) was covalently linked to CNBr-activated sepharose 6MB (Pharmacia Ltd.). Briefly, 1 g of CNBr-activated sepharose was reabsorbed and then prehydrolyzed for 3 h in 0.1 M carbonate-bicarbonate buffer pH 8.3 containing 0.5 M NaCl. The sepharose was mixed with 3 mg anti-IgE in 6 ml 0.1 M carbonate-bicarbonate buffer pH 7.0, containing 0.5 M NaCl. The sepharose was mixed with the solutions overnight at 4°C in an end-over-end mixer.

After the coupling the gel was washed and resuspended for 2 h in 1 M ethanolamine pH 9.0. The blocking reagent was washed away with 0.1 M carbonate-bicarbonate buffer pH 8.3, containing 0.5 M NaCl followed by 0.1 M acetate buffer pH 4.0, containing 0.5 M NaCl. This washing cycle was repeated two times,

after which the gel was suspended in Tris ACM-buffer. Protein analysis showed that approximately 90% of the protein was bound to sepharose 6MB. The gel suspension was poured into a small-size plastic column (0.9×7.5 cm, Pharmacia Ltd.). The histamine releasing property of anti-IgE-sepharose 6MB was tested on human leukocytes in batch-wise experiments and with the coupling conditions described above.

Affinity chromatography of cells on anti-IgE-sepharose 6MB

Five to 10 ml of cell suspension from stage 2 was loaded on to the column and allowed to pass through the column at a low flow rate. The flow rate was varied between 8 ml/h and 40 ml/h. Unbound cells were washed from the column with 30 ml Tris ACM at a flow rate of 40 ml/h. The specifically bound cells could either be eluted with Tris ACM at high flow rate or, when the cells were loaded on the column at a low flow rate, through mechanical agitation (5, 14).

Histamine assay

The spectrofluorometric technique of Shore et al. (13), with modifications by Lichtenstein et al. (7), was used for determination of histamine. The complete histamine values were obtained by treating the cell fractions with 0.2 ml 8% HClO_4 .

Protein assay

Protein determination of the column fractions was made using the method described by Lowry et al. (8).

Cell count

The method used for counting basophils has been described by Moore & James (9). The white blood cells were counted in a Bürker-chamber using a conventional laboratory method.

RESULTS

Chromatography on anti-IgE-sepharose and uncoupled sepharose

The anti-IgE-sepharose released histamine from human leukocytes when sepharose and cells were mixed and incubated at 37°C for 45 min.

When the cells are loaded on to the anti-IgE-sepharose 6MB column at a low flow rate, most of the basophils and some mononuclear cells are strongly bound to the sepharose. The unbound cells were easily eluted with Tris ACM-buffer, while the bound cells have to be eluted through mechanical agitation. The gel was removed from the column and suspended in Tris ACM solution. The suspension was stirred for a short time and the cells isolated from the supernatant.

The basophils bound to the sepharose could be seen when studying the column material in a microscope (Fig. 1).

A typical experiment is shown in Fig. 2. The total histamine values for each fraction have been plotted on the elution diagram. Most of the histamine is found in the last fraction, which indicated that the majority of basophils are bound to the sepharose. To determine whether the adsorption of basophils was specific or non-specific, reference-sepharose without bounded anti-IgE-sepharose was prepared. Reference-sepharose and anti-IgE-sepharose are compared in the experiment shown in Fig. 2. Some basophils are bound to the reference-sepharose but the anti-IgE-sepharose had much greater binding capacity.

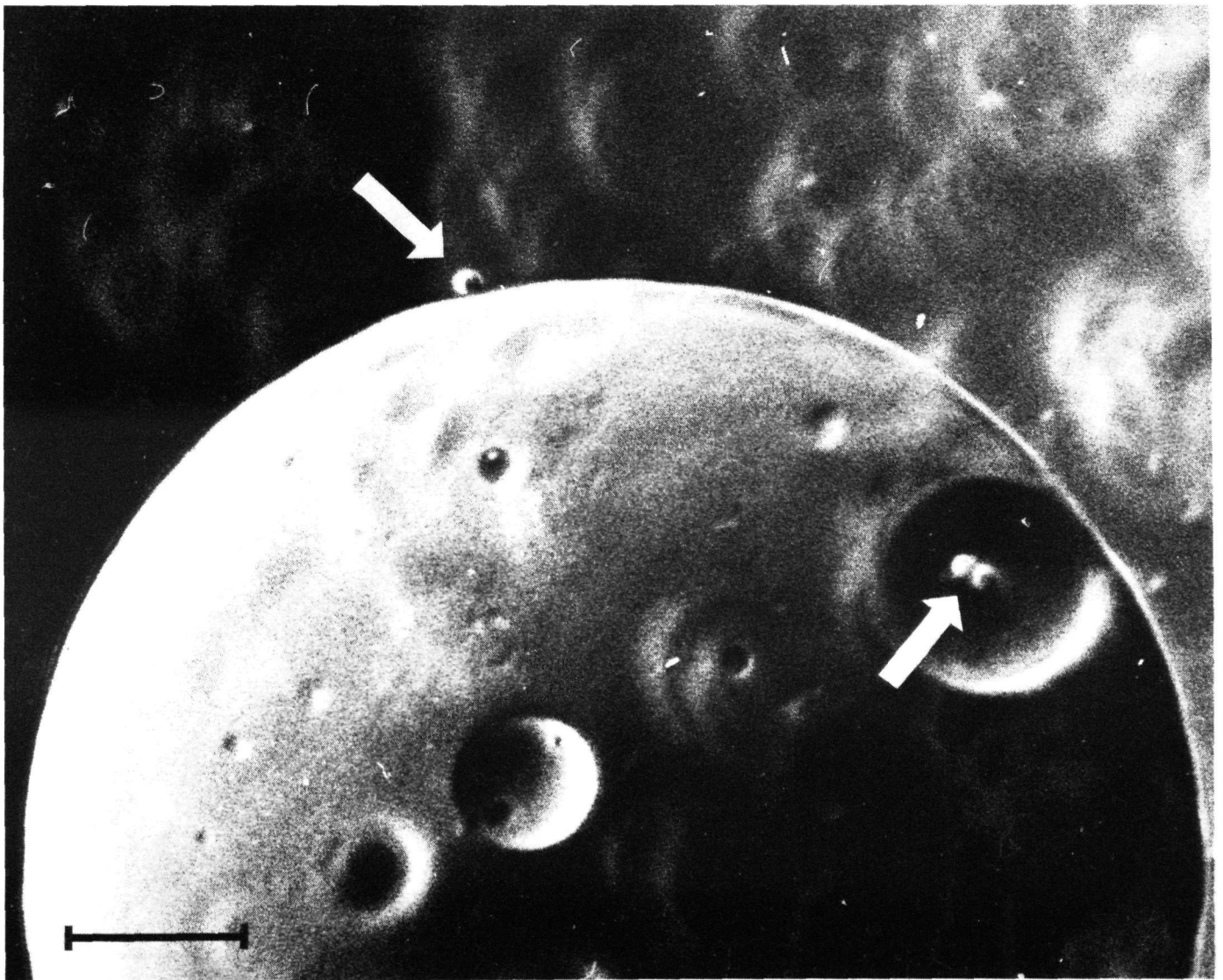


Fig. 1. Phase contrast photomicrograph of basophils bound to anti-IgE sepharose-6MB. The white arrows indicate cells attached to the sepharose. The black line indicates a distance of 50 μm .

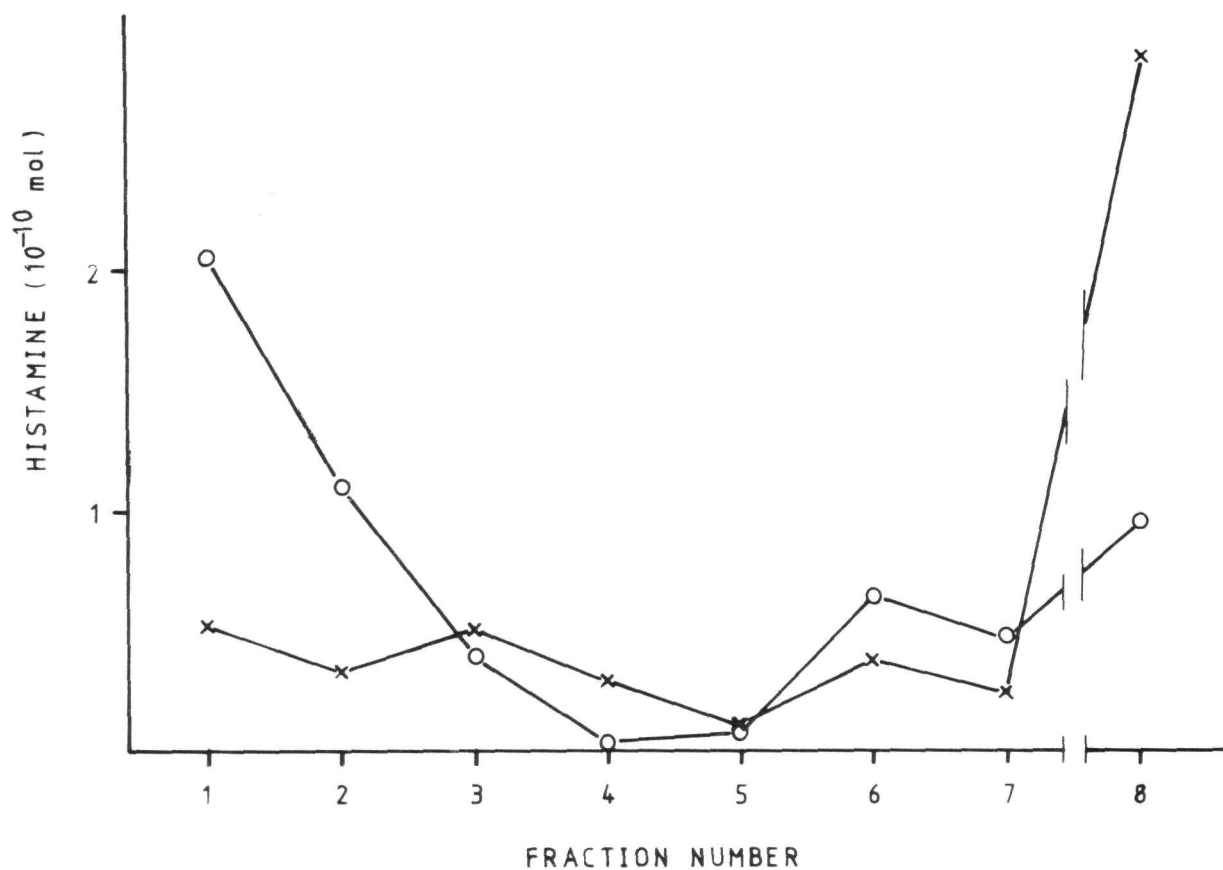


Fig. 2. Purification of human basophils on anti-IgE sepharose-6MB, (x—x), and reference-sepharose-6MB (o—o). Approx. 1.5×10^{-6} leukocytes were fractionated as described in Material and Methods. The flow rate was 20 ml/h when loading the column, and adjusted to 40 ml/h during elution. Fraction sizes were 5 ml. One representative experiment out of three.

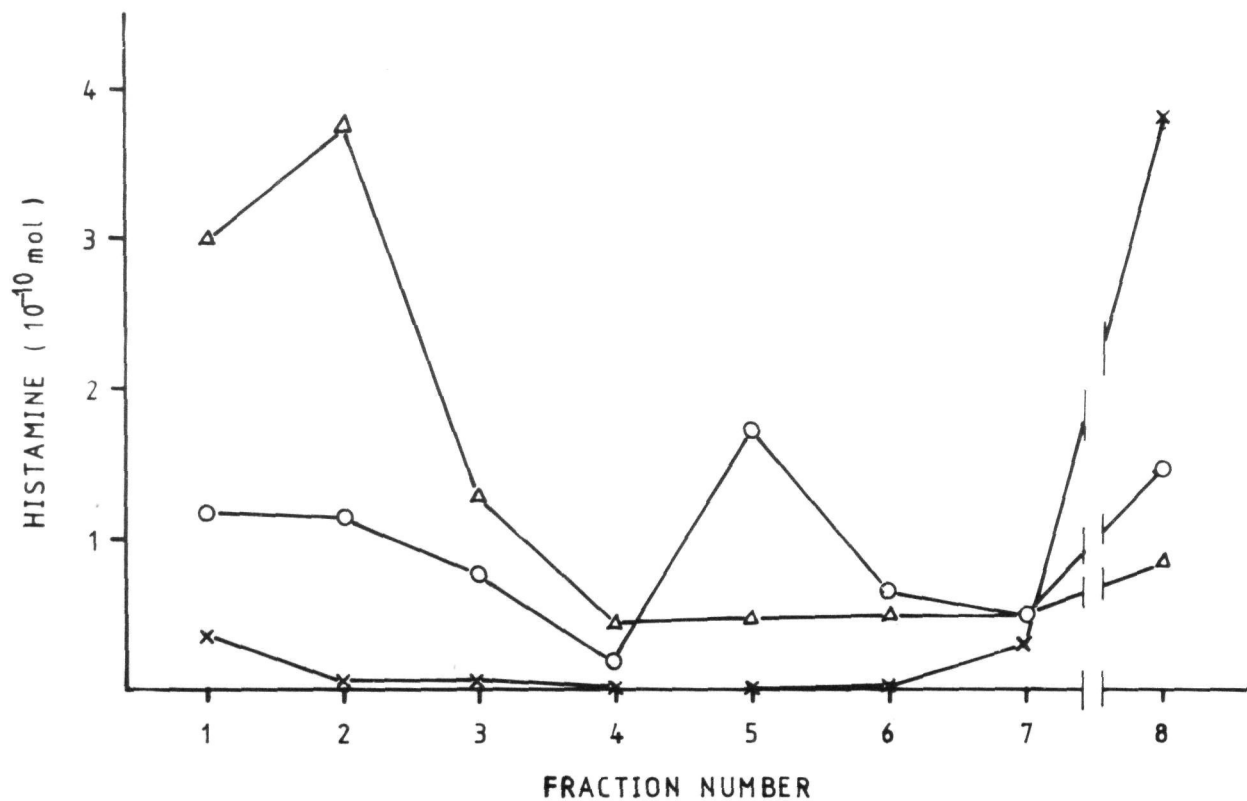


Fig. 3. Purification of human basophils on anti-IgE sepharose-6MB at different loading flow rates. a) Flow rate 8 ml/h (x—x). b) Flow rate 16 ml/h (o—o). c) Flow rate 38 ml/h (Δ—Δ). Approx. 1.5×10^{-6} leukocytes were fractionated in each experiment. Fraction sizes were 5 ml. One representative experiment out of four to six.

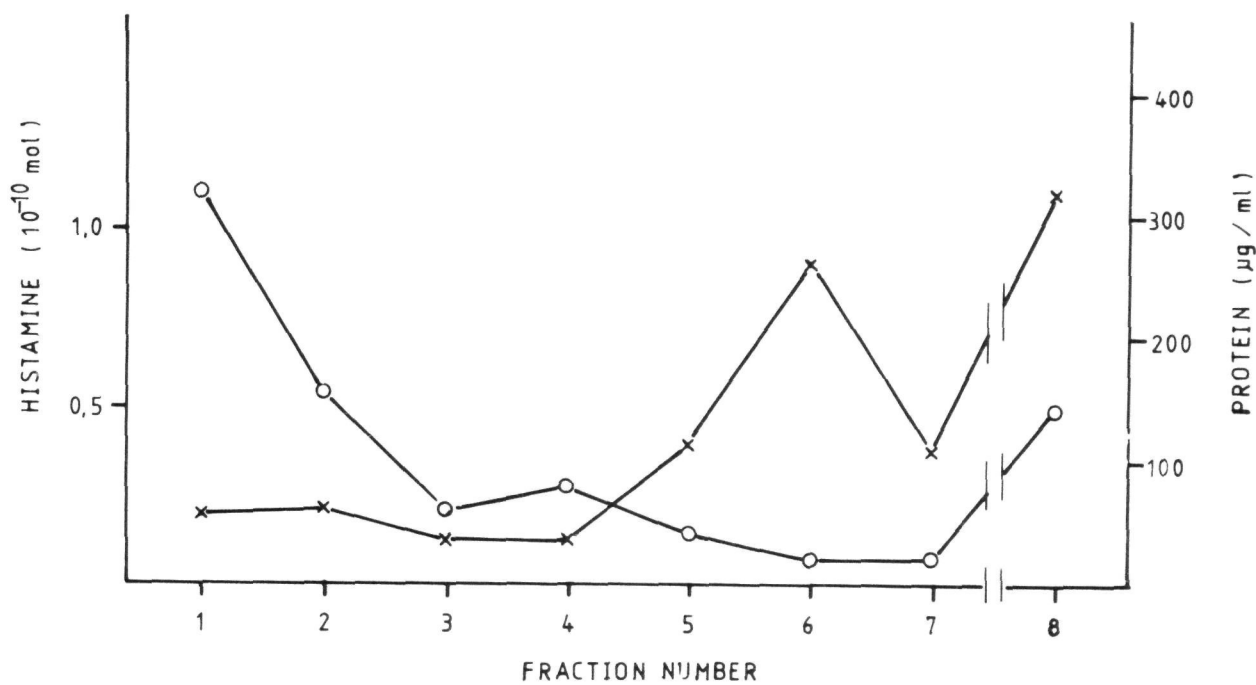


Fig. 4. Purification of human basophils on anti-IgE sepharose-6MB. Basophils (x—x) and protein concentration (O—O) are plotted in the diagram. Approx. 1×10^{-6} leukocytes were fractionated. The initial flow rate was 11 ml/h. Fraction sizes were 5 ml. One representative experiment out of five.

Chromatography on anti-IgE-sepharose with selective elution of basophils

The effect of different flow rates is shown in Fig. 3. At the low flow rate most of the basophils are bound to the sepharose, while at the higher rates most of the cells are eluted at the start of chromatography. If the slow rate is enhanced and the loaded cell suspension diluted, it is

possible to achieve a selective elution of basophils. Fig. 4 shows a typical run where basophils were eluted in fractions F5–F7. Some basophils were still bound to the sepharose and could be eluted mechanically. The protein curve shows that most of the cells were eluted at the start of chromatography. In the fractions F5–F7 only a very small amount of protein is seen, which indicates that the purity of baso-

Table 1
Isolation of basophil leukocytes from human peripheral blood. Purity in percent of total leukocytes

	Peripheral blood	Stage 1+2	Selective elution		Mechanical elution	
			Stage 3	Stage 1+2+3	Stage 3	Stage 1+2+3
Purification	—	2.5–3.5	25–40	65–100	10–20	25–35
Purity (%)	0.5–1.5	1.3–5.3	—	80–95	—	40–60
Recovery (%)	100	66–82	30–40	20–35	42–54	30–45

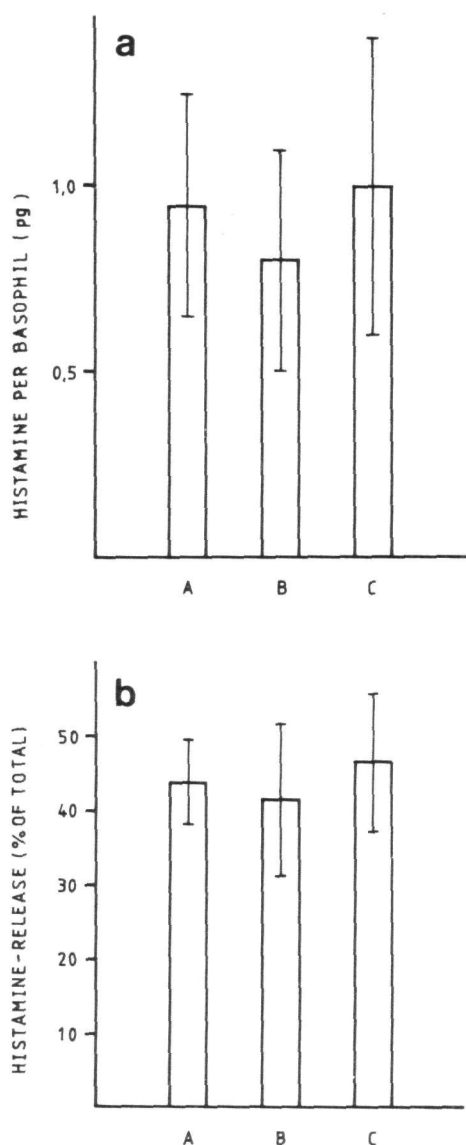


Fig. 5a. Amount of histamine per basophil after stage 1 (A), stage 2 (B) and stage 3 (C). Mean $\pm$ SEM ($n = 4$).

Fig 5b. Functional test of separated basophils. The cells were incubated with optimal doses of anti-IgE. Fractionated basophils, stage 3 (C) released histamine in a normal way compared with cells in stage 1 (A) and stage 2 (B). Mean $\pm$ SEM ($n = 4-5$).

phils is very high. This is confirmed by the cell counting.

Degree of purification and recovery during cell separation

The results of five cell separations are summarized in Table 1. In the first two stages of the separation the purification was 2–3-fold with a recovery of 65–80%.

In the final chromatography the purification was 10–20-fold and the recovery approximately 50%, when the cells were eluted mechanically.

When the basophils were selectively eluted the final purity was as high as 80–95% with a recovery of approximately 30%.

Separation of basophils from various persons did not give cell fractions of equal purity. The variation could be rather high, as seen by variation of final purity in Table 1. This can be explained by a different initial purity and antigen-binding capacity. The day-to-day variation in the same person was rather low, although the absolute amount of histamine could vary.

Functional test of separated cells

The basophils separated by the described method showed no loss of histamine (Fig. 5a) and released histamine, when exposed to anti-IgE (Fig. 5b).

DISCUSSION

The experiments presented in this work provide a method for purification of human basophils. The purity obtained was very high, up to 95%, but the recovery was rather low. The described anti-IgE-sepharose column works with both specific and non-specific adsorption. In order to obtain specific binding sites, the coupling procedure for anti-IgE must be performed exactly by the specified method. The antigen-binding sites on the sepharose, which were free to interact with the IgE-coated cells, were tested by determining the histamine releasing effect from human leukocytes at 37°C. Prehydrolysis for 3 h was necessary for obtaining an acceptable density of active antigen-binding sites. The unspecific binding forces of sepharose are small compared with glass and plastic beads, but even in the reference-sepharose (Fig. 2) a fractionation due to weak non-specific forces could be observed. Ishizaka et al. (6) have used non-specific forces of a glass-bead column to prepare a basophil-rich fraction. We have also tested this separation technique (Toll, unpublished results), but the non-specific binding properties of glass-beads attracted a very mixed population of leukocytes, so the degree of purification was not satisfied.

The flow rate through the anti-IgE-sepharose

column was a very important factor for the adsorption and desorption of cells to the specific binding sites. The cells were bound rather tightly to the sepharose beads at a low flow rate (Fig. 3). The forces that enable binding at a low flow rate have been discussed by Wigzell (14). He suggested that a specific but weak force retards the flow rate of the individual cells through the column. Secondly, this decreased flow rate in itself will then greatly enhance the possibility that the cell will be bound to the beads via the non-specific forces. By increasing the flow rate it was possible to perform a more specific fractionation, because the non-specific forces of sepharose were diminished. With a moderate flow rate it was also possible to elute a very pure basophil fraction (Table 1).

The separated cells appeared normal. One might expect that anti-IgE-sepharose would release histamine from the basophils and make the cells desensitized. However, no histamine was released during column fractionation and the separated basophils released histamine when incubated with anti-IgE at 37°C (Fig. 5). It seems obvious that the temperature is of great importance. When the separation was carried out at room temperature, or lower, no histamine release occurred under fractionation and the cells were not desensitized.

The purity obtained with this procedure is comparable with that obtained by Day (3). We have tested the method of Day, but in our hands we could not obtain the described purity and recovery of basophils. The affinity chromatography method described here yielded a more useful basophil fraction. It appeared that the basophils separated by this method were normal and were found to be reactive with anti-IgE in subsequent tests. We have evidence that the basophil purified by this method will afford a useful system for studying some of the biochemical mechanisms of histamine release and other aspects of basophil function.

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REFERENCES

1. Bennich, H. & Johansson, S. G. O.: Structure and function of human immunoglobulin E. *Adv. Immunol.* **13**, 1-55, 1971.
2. Böyum, A.: A one stage procedure for isolation of granulocytes and lymphocytes from human blood. *Scand. J. Clin. Lab. Invest.* **97**, Suppl. 21, 51-76, 1968.
3. Day, R. P.: Basophil leukocyte separation from human peripheral blood: a technique for their isolation in high purity and high yield. *Clin. Allergy* **2**, 205-212, 1972.
4. Dvorak, H. F., Sevaggio, S. S., Dvorak, A. M., Colvin, R. B., Lean, D. B. & Rypysc, J.: Purification of basophilic leukocytes from guinea pig blood and bone marrow. *J. Immunol.* **113**, 1694-1702, 1974.
5. Ghetie, V., Mota, G. & Sjöquist, J.: Separation of cells by affinity chromatography on SpA-sepharose-6MB. *J. Immunol. Methods* **21**, 133-141, 1978.
6. Ishizaka, T., Soto, C. S. & Ishizaka, K.: Mechanisms of passive sensitization. III. Number of IgE molecules and their receptor sites on human basophil granulocytes. *J. Immunol.* **3**, 500-511, 1973.
7. Lichtenstein, L. M. & Osler, A. G.: Studies on the mechanisms of hypersensitivity phenomena. IX. Histamine release from human leukocytes by ragweed pollen antigen. *J. Exp. Med.* **120**, 507-530, 1964.
8. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J.: Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265-275, 1951.
9. Moore, J. E. & James, G. W.: A simple direct method for absolute basophil leukocyte count. *Proc. Soc. Exp. Biol. Med.* **82**, 601-603, 1953.
10. Murphy, R. F., Buchanan, K. D. & Elmore, D. T.: Isolating of glucagon-like immunoreactivity of gut by affinity chromatography on anti-glucagon antibodies coupled to sepharose 4B. *Biochim. Biophys. Acta* **303**, 118-127, 1973.
11. Pruzansky, J. J. & Patterson, R.: Decrease in basophils after incubation with specific antigens of leukocytes from allergic donors. *Int. Arch. Allergy* **38**, 522-526, 1970.
12. Rabinowitz, Y.: Separation of lymphocytes, polymorphonuclear leukocytes and monocytes on glass columns, including tissue culture observations. *Blood* **23**, 811-828, 1964.
13. Shore, P. A., Burkhalter, A. & Cohn, Jr., V. H.: A method for the fluorometric assay of histamine in tissues. *J. Pharmacol. Exp. Ther.* **127**, 182-186, 1959.
14. Wigzell, H.: Specific affinity fractionation of lymphocytes using glass or plastic bead columns. *Scand. J. Immunol.* **5**, Suppl. 5, 23-30, 1976.

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