

Detection of human leucocytes by cyclic voltammetry and its application to monitoring of allergic reaction*

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Abstract: Cyclic voltammetry was applied to the detection of human leucocytes and the monitoring of allergic reactions. A basal plane pyrolytic graphite electrode with attached leucocytes on a porous nitrocellulose membrane filter was employed as a working electrode. An anodic peak current appeared at 0.33 V versus the saturated calomel electrode (SCE) when the potential of the working electrode was scanned in the range of 0–1.0 V versus SCE. This peak current was attributed to the electrochemical oxidation of serotonin. When egg white was added to leucocytes obtained from patients who were allergic to egg, the peak current decreased owing to degranulation of leucocytes leading to serotonin release. The peak current decreased with increasing allergen concentration in the range of 5–50 $\mu\text{g ml}^{-1}$. Leucocytes did not respond to other allergens such as soybean, milk and dinitrophenylated bovine serum albumin (DNP-BSA).

Keywords: leucocyte, cyclic voltammetry, serotonin, immunoglobulin E, allergic reaction.

INTRODUCTION

The radioimmunosorbent test (RIST) (Green-Graif & Ewan, 1987; Merrett & Merrett, 1987; Walsh *et al.*, 1987), radioallergosorbent test (RAST) (Kelly *et al.*, 1980), and skin test (Dreborg *et al.*, 1986; Green-Graif & Ewan, 1987) have been used for sensing allergic reactions. However,

RIST and RAST are time-consuming and demand complicated procedures, giving results independent of clinical symptoms. The skin test is dangerous because it may produce anaphylaxis in man by means of serum antibodies. A simple and safe method is still required for the detection of allergic reactions.

Recently, we have developed a method for the electrochemical detection of the allergic reaction using the rat basophilic leukemia (RBL-1) based on cyclic voltammetry at a basal plane pyrolytic graphite electrode (Matsunaga *et al.*, 1989).

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Serotonin present in the cell is electrochemically oxidized and used as a marker (Fig. 1). RBL-1 cells were sensitized with monoclonal mouse anti-dinitrophenylated (DNP) immunoglobulin E (IgE). When dinitrophenylated bovine serum albumin (DNP-BSA), as a model allergen, was added to anti-DNP-sensitized RBL-1 cells, the peak current of the cyclic voltammogram decreased owing to degranulation of RBL-1 cells, leading to serotonin release.

If this system is applied to the detection of food and pollen allergy, IgE should be prepared. However, it is time-consuming and expensive to prepare IgE for each allergen. Moreover, RBL-1 cells are not necessarily sensitized with monoclonal or polyclonal IgE. Therefore, leucocytes prepared directly from patients were used in this work. Electrochemical detection of human leucocytes was carried out using an electrode system consisting of a graphite electrode and a porous nitrocellulose membrane filter to trap leucocytes. This system was applied to the detection of food allergies.

MATERIALS AND METHODS

Materials

Sodium 2,4-dinitrobenzenesulfonate was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Bovine serum albumin (BSA), 5-hydroxytryptamine hydrochloride (serotonin), and compound 48/80 (a condensation product of *N*-methyl-*p*-methoxyphenethylamine with formaldehyde) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Heparin calcium solution was a gift from Kodama Co., Ltd. (Tokyo, Japan). Mono-poly resolving medium was obtained from Flow Laboratories (North Ryde, Australia). Allergen extracts (egg white, soybean and cow's milk) were prepared as reported previously (Matsunaga *et al.*, 1989). The protein concentration of allergen extract was determined by the Lowry method (Lowry *et al.*, 1951). Other reagents were commercially available analytical reagents or

laboratory grade materials and were used as received. Deionized distilled water was used in all procedures.

Fractionation of whole blood

Heparin calcium solution (0.8 ml, 1000 U/ml) was added to 40 ml of whole blood obtained from patients with a food allergy and from normal adults. Samples were incubated at 25°C for 30 min after the addition of 5 ml of 2% methyl cellulose solution. Erythrocytes were obtained as a precipitate. The supernatant was then centrifuged at 120g for 1 min, to obtain leucocytes which formed a precipitate.

Separation of neutrophils and lymphocyte-monocyte mixture from whole blood

The whole blood containing heparin (5 ml) was added to 3 ml of mono-poly resolving medium (MPRM). The sample was centrifuged at 300g for 30 min. The uppermost layer containing plasma was removed using a pipette. The next layer containing the lymphocyte-monocyte mixture and the layer of neutrophils were obtained and washed with phosphate buffered saline (PBS; 1.5 mM KH_2PO_4 , 7.3 mM Na_2HPO_4 , 137 mM NaCl, 2.7 mM KCl, pH 7.4), respectively.

Apparatus

The electrode system consisted of a basal plane pyrolytic graphite (BPG) electrode (surface area, 0.19 cm²; Union Carbide Corp., New York), an auxiliary electrode (platinum wire), and a membrane filter for retaining leucocytes (Matsunaga *et al.*, 1989). The reference electrode was a saturated calomel electrode (SCE). Cyclic voltammograms were obtained using a potentiostat (HA301, Hokuto Denko, Tokyo, Japan), a function generator (HB104, Hokuto Denko, Tokyo, Japan), and an X-Y recorder (F35, Riken Denshi, Tokyo, Japan). After each run, the graphite electrode was polished with emery paper.

Procedure for the cyclic voltammetry

Separated leucocytes and erythrocytes were washed twice with cold (4°C) PBS. Then, leucocytes and erythrocytes were resuspended in 1 ml of PBS to a density of 10⁶ cells ml⁻¹ and 10⁷

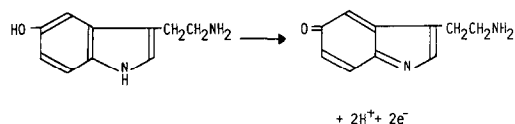


Fig. 1. The reaction scheme of serotonin.

cells ml^{-1} , respectively. The lymphocyte-monocyte mixture and neutrophils were also resuspended in 1 ml of PBS to a density of 10^6 cells ml^{-1} . The cells were fixed on the membrane filter by filtration using an aspirator. The recovery of cells fixed on the membrane filter was measured by trypan blue and microscopic observation and 90% of leucocytes on the surface of membrane filter were viable. The cells on the membrane filter were attached to the basal plane pyrolytic graphite. Cyclic voltammetry was run in the range of 0–1.0 V versus SCE in 10 ml of PBS (pH 7.4).

Procedure for the detection of allergic reaction

Leucocytes were centrifuged at 120g for 1 min and washed twice with Tris-A buffer (25 mM Tris-base, 120 mM NaCl, 5 mM KCl, and 0.3 mg ml^{-1} BSA, pH 7.6). They were resuspended to a density of 1.0×10^6 cells ml^{-1} in 1 ml Tris-ACM buffer (Tris-A buffer containing 0.6 mM CaCl_2 and 1.0 mM MgCl_2). Then allergen extract was added to the leucocyte suspension to a final concentration of $10 \mu\text{g ml}^{-1}$. After the cells had been incubated for 1 h at 37°C in 5% CO_2 , the cell suspension was poured onto the membrane filter and used for the cyclic voltammetry as described above. The fluorescence intensity of serotonin was determined using a fluorescence spectrophotometer (F-1200, Hitachi, Tokyo, Japan) with the excitation wavelength set at 298 nm and the emission wavelength at 330 nm. Degranulation of cells was confirmed by microscopy (Weill & Renoux, 1982).

Sonication of leucocytes

Leucocytes (1.0×10^6 cells) were suspended in 10 ml of PBS (pH 7.4) and then disrupted using an ultrasonic disrupter (UR-200P, TOMY Seiko Co., Ltd., Tokyo, Japan) operated for 5 min at 0°C over five times. The sonicated cells were centrifuged at 4°C and 120g, and the exudate of sonicated cells was obtained. Collected cells were resuspended in 1 ml of PBS and cell suspension was dropped onto the membrane filter and used for measurement as described above.

Degranulation of leucocytes

Leucocytes (1.0×10^6 cells) were suspended in 9 ml of PBS (pH 7.4). Compound 48/80 solution

(1 ml, $100 \mu\text{g ml}^{-1}$) was added to the cell suspension, which was then incubated for 1 h at 37°C in 5% CO_2 . The degranulated cells were centrifuged at 120g, and the eluent of degranulated cells was obtained. Serotonin in the supernatant was analyzed as described above.

RESULTS AND DISCUSSION

Cyclic voltammetry of leucocytes and erythrocytes

Whole blood from patients with a food allergy and from normal adults was divided into leucocytes and erythrocytes. Figure 2 shows the cyclic voltammograms of leucocytes and erythrocytes from a normal adult (23 years old, male). Anodic waves appeared at 0.33 V and 0.68 V versus SCE for leucocytes and 0.68 V versus SCE for erythrocytes. The peak current at 0.33 V versus SCE was $0.36 \mu\text{A}$ for 10^6 cells. Upon scan reversal, no corresponding reduction peak was obtained. The electrode reactions of both leucocytes and erythrocytes were irreversible. The peak current of patients and other normal adults appeared at the same potential. The oxidation peak current of leucocytes was found to increase linearly with the square root of the scan rate as expected for a totally irreversible reaction. The peak currents at 0.33 V versus SCE increased linearly with increasing leucocyte numbers up to 2.0×10^6 cells (Fig. 3). Recently, it was found that the peak current of the cyclic voltammograms of

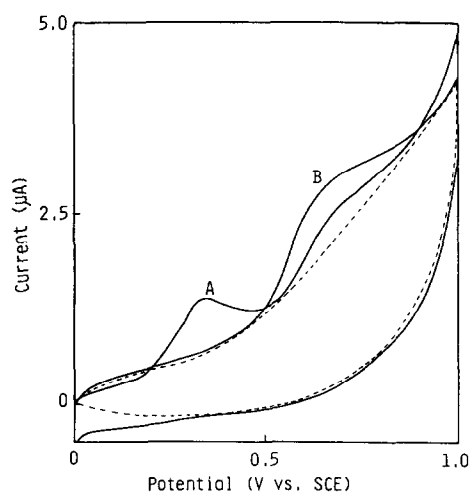


Fig. 2. Cyclic voltammograms of (A) leucocytes (1.0×10^6 cells), (B) erythrocytes (1.0×10^7 cells) and PBS only (broken line). The scan rate was 10 mV s^{-1} . The measurements were performed in 10 ml of PBS (pH 7.4).

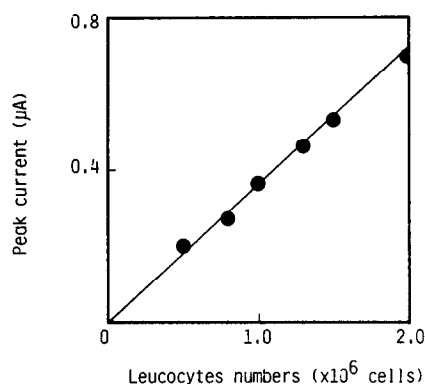


Fig. 3. Relationship between peak current at 0.33 V versus SCE and leucocyte numbers. The scan rate was 10 mV s^{-1} . The measurements were performed in 10 ml of PBS (pH 7.4).

lymphocyte, macrophage, ascites tumor, erythrocyte, yeasts, and bacteria was observed at 0.65–0.74 V versus SCE which was attributed to the oxidation of coenzyme A (CoA) present in the cells (Matsunaga & Namba, 1984a, b; Matsunaga & Nakajima, 1985; Matsunaga *et al.*, 1989). Therefore, the peak current of leucocytes at 0.68 V versus SCE is due to electrochemical oxidation of CoA. It was also reported that the RBL-1 cell showed a peak current at 0.33 V versus SCE which was attributed to the electrochemical oxidation of serotonin. The peak current of leucocytes at 0.33 V versus SCE was due to the electrochemical oxidation of serotonin in the cell. The electrochemical oxidation of leucocytes at 0.33 V was studied. Figure 4 shows the effect of pH on peak potentials. The slope of peak potential versus pH line was 60 mV in the range of

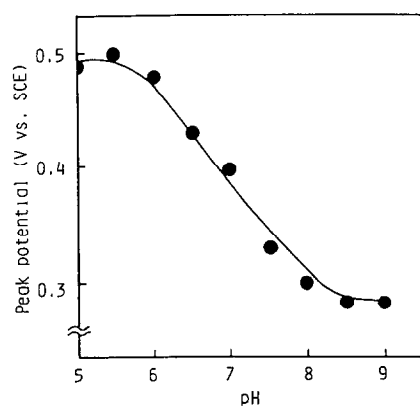


Fig. 4. Peak potential as a function of pH. Leucocytes (1.0×10^6 cells) were fixed onto the membrane filter. The scan rate was 10 mV s^{-1} . The pH of the solution was adjusted with 0.1 N HCl and 0.1 N NaOH solutions.

pH 6.0–8.0. These results indicate that two protons are transferred in this range, as previously reported (Matsunaga *et al.*, 1989), because the electrode reaction of serotonin was also two electron transfer reaction in this range (Wrona & Dryhurst, 1987). The pH of the sample solution was adjusted to 7.4 for the detection of allergic reaction.

The leucocytes from normal adults consist of 62% neutrophils, 0.5% basophils, 2.5% eosinophils, 5% monocytes and 30% lymphocytes. Therefore, leucocytes from normal adults were fractionated into neutrophils and lymphocyte-monocyte mixture. These are the major components of leucocytes, and form the main contribution to the current generation of leucocytes. Figure 5 shows the cyclic voltammograms of neutrophils and a lymphocyte-monocyte mixture, respectively. An anodic wave also appeared at 0.33 V versus SCE for neutrophils. The peak current at 0.33 V versus SCE was $0.58 \mu A$ for 1.0×10^6 cells. However, no peak current was obtained from the lymphocyte-monocyte mixture at around 0.33 V versus SCE. This means that the peak current of leucocytes at 0.33 V versus SCE was almost all due to neutrophils.

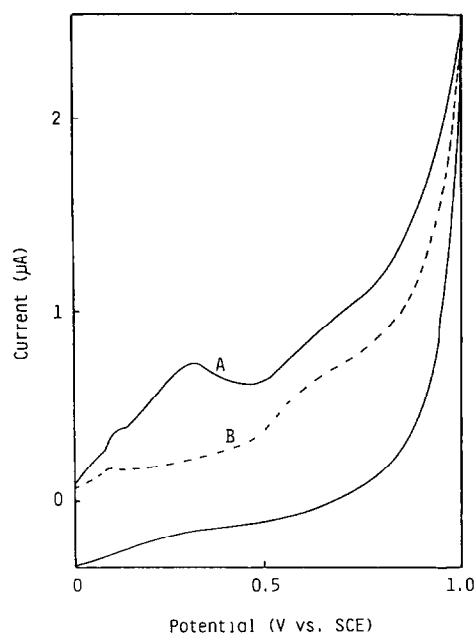


Fig. 5. Cyclic voltammograms of (A) neutrophils (1.0×10^6 cells) and (B) lymphocyte-monocyte mixture (1.0×10^6 cells). The conditions of measurements were the same as described in Fig. 2.

Mechanism of electrochemical reaction in leucocytes

Recently, it was found that the peak current of serotonin is obtained at 0.33 V versus SCE at pH 7.0 by using a BPG electrode. It was reported that the peak potential appeared at 0.29–0.34 V versus SCE around pH 7.0 when using carbon fiber microelectrodes (Wrona & Dryhurst, 1987) and pyrolytic graphite electrodes (Ponchon *et al.*, 1979). The peak potential of leucocytes was similar to that obtained from serotonin in our experiments (Matsunaga *et al.*, 1989) and in previous reports (Ponchon *et al.*, 1979; Wrona & Dryhurst, 1987). On the other hand, the peak current was not obtained from other mediators of anaphylaxis such as histamine. Moreover, no corresponding reduction peak was obtained with serotonin.

In order to verify the contribution of serotonin to current generation, intracellular serotonin was released by sonication, and the current of sonicated leucocytes, together with the serotonin concentration in the solution, was measured. The concentration of serotonin released from leucocytes was determined using a fluorescence spectrophotometer. Leucocytes (10^6 cells) were suspended in 10 ml of PBS and this suspension was incubated at 25°C. Figure 6 shows the relationship between the peak current of leucocytes and the serotonin concentration in the solution. The peak current at 0.33 V versus SCE decreased gradually. The peak current decreased with increasing released serotonin concentration

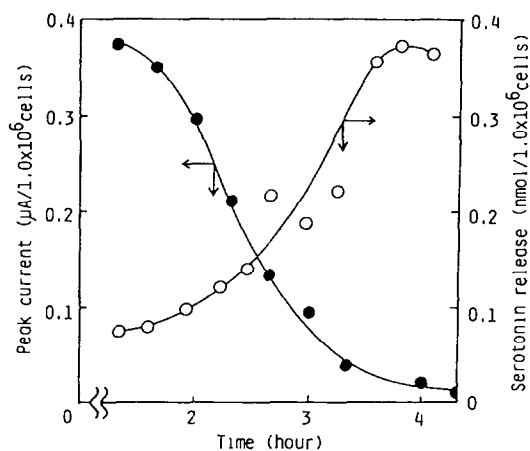


Fig. 6. Relationship between (●) peak current and (○) serotonin release from human leucocytes (1.0×10^6 cells) suspended in 10 ml of PBS (pH 7.4). The scan rate was 10 mV s^{-1} .

in the solution. After 4 h, the amount of serotonin released from leucocytes was $0.37 \text{ nmol per } 10^6$ cells. It was assumed that sonication of leucocytes caused the release of serotonin.

Leucocytes were then degranulated with compound 48/80. This compound is employed as the inducer of degranulation. Figure 7 shows cyclic voltammograms of leucocytes and of degranulated leucocytes. The peak current at 0.33 V versus SCE decreased from $0.27 \mu\text{A per } 10^6$ cells to $0.05 \mu\text{A per } 10^6$ cells. Decrease in the serotonin content of leucocytes agreed with the amount of serotonin released.

Detection of food allergy reaction using leucocytes

Allergic reactions are included in immunological responses. IgE antibodies, which are secreted by activated B lymphocytes, are responsible for the allergic reactions. The Fc region of the IgE molecule binds to specific receptor proteins on the surface of basophilic leucocytes in the blood. These bound IgE molecules then serve as receptors for allergens. Allergens cross-link the membrane-bound IgE antibodies with complementary allergen-binding sites, thereby triggering the cells to secrete chemical mediators such as histamine. These chemical mediators make neutrophilic leucocytes release histamine and serotonin. Histamine and serotonin cause dilation and increased permeability of blood vessels and are largely responsible for the clinical manifestations of such allergic reactions as

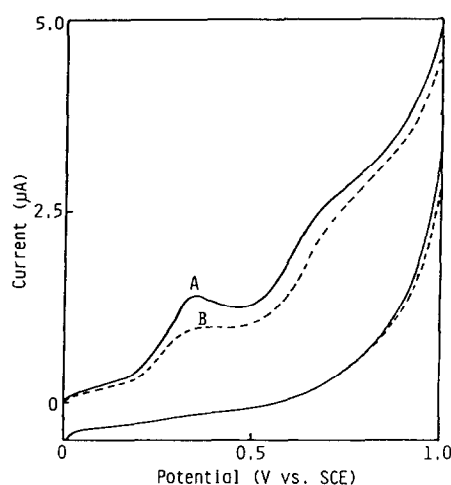


Fig. 7. Cyclic voltammograms of (A) intact leucocytes and (B) degranulated leucocytes with compound 48/80. 1.0×10^6 cells were used in each case. Other experimental conditions were the same as described in Fig. 2.

TABLE 1

Peak current of leucocytes and serotonin release in the solution when egg white, milk and soybean were added to leucocytes from a patient with an egg allergy

Extract of allergen (10 $\mu\text{g ml}^{-1}$)	Peak current decrease ($\mu\text{A per } 10^6 \text{ cells}$)	Serotonin release (nmol per 10^6 cells)
Egg white	0.08 ± 0.01	0.57 ± 0.05
Milk	0.02 ± 0.02	0.08 ± 0.04
Soybean	0.05 ± 0.02	0.20 ± 0.05

pollenosis, asthma and hives. Basophilic leucocytes have IgE receptors on their surface. Therefore, the decrease in peak current and the serotonin release were examined when allergen extract was added to leucocytes. Leucocytes obtained from patients with egg allergy (5 years old, male) were incubated with egg white as an allergen for 1 h. The patients showed IgE values in their blood of 16.0 U ml^{-1} , 6.7 U ml^{-1} and 2.6 U ml^{-1} for egg white, milk and soybean, respectively. Table 1 shows the peak currents obtained from leucocytes and the amount of serotonin released into solution when egg white, milk and soybean were added to leucocytes of egg allergy patients. After the incubation, the peak current decreased from $0.27 \mu\text{A per } 10^6 \text{ cells}$ to $0.19 \mu\text{A per } 10^6 \text{ cells}$. However, the decreases in peak current were lower when milk and soybean were added. Various allergen extracts such as pollen, dust and DNP-BSA did not show a peak current decrease. Serotonin release of leucocytes occurred by cross-linking with the corresponding allergen in egg extract. 0.57 nmol of serotonin was released from 10^6 cells after egg white addition. When milk and soybean was added to the leucocytes, released serotonin was $0.08 \text{ nmol per } 10^6 \text{ cells}$ and $0.20 \text{ nmol per } 10^6 \text{ cells}$, respectively. Leucocytes from normal adults were incubated with egg white for 1 h. After the incubation, the peak current decrease was not observed. Other allergen extracts (milk and soybean) did not show a peak current decrease. Leucocytes from another patient suffering from a milk allergy were also incubated with egg white. In this case, the peak current of leucocytes did not respond to other allergens. The response of leucocytes taken from patients to egg allergy was specific to egg white. Leucocytes from another patient suffering from a milk allergy were also used for the

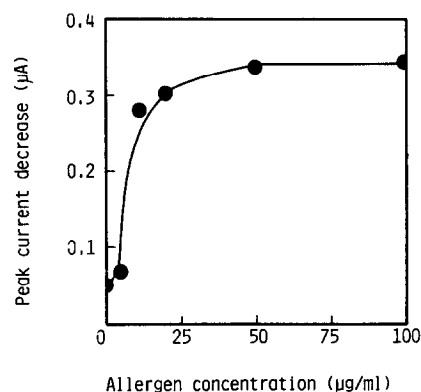


Fig. 8. Relationship between allergen (egg white) concentration and peak current of leucocytes taken from patient to egg allergy. Cells 5.0×10^5 were used in each case. Other experimental conditions were the same as described in Fig. 2.

serotonin release experiment. The amount of released serotonin was $0.050 \text{ nmol per } 10^6 \text{ cells}$ when milk was added to the leucocytes suspension. When soybean and egg white were added, 0.020 and $0.018 \text{ nmol per } 10^6 \text{ cells}$ of serotonin were released into solution, respectively. The IgE values of the patients were 1.0 U ml^{-1} , 0.6 U ml^{-1} and 0.4 U ml^{-1} for milk, soybean and egg white, respectively. The peak current of leucocytes and the amount of serotonin released correlated well with IgE value for each allergen. The peak current of leucocytes decreased and the amount of leucocytes released went up with increasing allergen (egg white) concentration in the range of $5\text{--}50 \mu\text{g ml}^{-1}$ when the leucocytes of patients with an egg allergy were employed (Fig. 8). The peak currents and the fluorescence intensity of serotonin were reproducible with a relative error of 5%. These results suggest that specific serotonin release of allergic reaction can be detected from the peak current obtained using cyclic voltammetry.

Further studies will be directed towards applying this detection system to other food allergy and pollenosis.

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