THREE-DIMENSIONAL OBSERVATION OF THE RADIOACTIVE
CONCAVALIN A RECEPTORS ON THE SURFACE OF
HUMAN RED BLOOD CORPUSCLES UTILIZING
SCANNING ELECTRON MICROSCOPE
AUTORADIOGRAPHY

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Autoradiography combined with scanning electron microscopy (SEM-ARG) was applied to visualize the Con A receptor sites of human red blood corpuscles. Prepared erythrocytes on a glass slide were coated with emulsion by a dipping technique after evaporation by carbon under clean vacuum condition with a cooling trap of liquid nitrogen. Silver halide crystals in the emulsion were distributed homogeneously on the surface of the erythrocytes. In these experiments, emulsion degelatinized by centrifugation seemed superior to normal emulsion. The remaining gelatin was removed by distilled water at 45°C after development.

Electroconductive staining with tannic acid and osmium tetroxide was effective in obtaining good electron contrast and also in preventing artifacts due to the autoradiographic procedure. The number of developed silver grains on the surface of the red blood cells was proportional to the length of exposure time. After exposure for 8 weeks, the average number of grains was calculated to be 312.46 ± 76.07 (^3H -Con A-labeled specimen) or 372.58 ± 106.05 (^{125}I -Con A-labeled specimen) per single red blood corpuscle face. The SEM image of the grains was spherical or filamentous, so it was easy to distinguish the grains from the red blood cell surface. On the specimens labeled with ^3H - or ^{125}I -Con A, a large number of developed silver grains were distributed homogeneously on the surface of the red blood cells, but on the cells labeled with the same radioactive Con A containing α -methyl-d-mannoside, there were almost no grains present on the surface. Therefore, the distribution of the developed silver grains probably represents Con A receptor sites.

SEM-ARG seems to be a valuable method for the visualization of receptors on the cell surface, even though several problems still remain.

Autoradiography (ARG) is a very useful technique not only for research on the metabolic pathways of biological materials such as nucleic acids, amino acids, and vitamins but also for the detection of hormone and neuromuscular activators, i.e. acetylcholine receptors. The method has been utilized extensively from macroscopic to transmission electron microscope (TEM) level (29). However, there seems to be a limit to the observation of tracer distribution on the cell surface, as it is difficult to demonstrate three-dimensional structures.

Recently, scanning electron microscopy combined with ARG (SEM-ARG) has been used by some investigators to study the relationship between the structure and

the function of the cell or the detection of receptors on the cell surface. Paul *et al.* (1970) first suggested the possibility that ARG was applicable to SEM studies on the basis of the evaluation of the distribution of ^3H -auxiliary on a cellulose fiber (33). Hodges and Muir (1974) reported SEM-ARG done with paraffin sections of bladder explants incubated with ^3H -thymidine (11). Sudár and Csaba (1978) applied SEM-ARG to the three-dimensional demonstration of ^{125}I -albumin or ^{125}I - T_3 on thymus and *tetrahymena* cultured cells (40). Junger and Bachmann (1980) observed the receptor sites of ^{125}I -insulin on free hepatocytes or leucocytes (14).

These results have suggested the usefulness of SEM-ARG for three-dimensional research on the location of receptors or the distribution of metabolic substances on the cell or tissue surface.

We also (1979, 1981, 1982) have reported previously an application of ARG to SEM study (21, 41, 42). In the present study, we use ^3H - and ^{125}I -Concanavalin A (Con A), and discuss the visualization and distribution of the receptors on the surface of human red blood corpuscles, which have been extensively studied by biochemical (6, 13, 35, 38) and histochemical (1, 12, 32, 36, 39) methods.

MATERIALS AND METHODS

1) Cell Preparation

Human blood was obtained from the basilic vein with a heparinized syringe. The blood cells were centrifuged for 5 min at 1,000 rpm after 30 μl of the blood was rinsed with physiologic saline solution, and this procedure was repeated twice. The rinsed blood cells were prefixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) at pH 7.3 for 30 min at 4°C. The fixed sediments were resuspended in the same PB and rinsed twice after centrifugation. The sediments were blocked with 0.05 M L-lysine (3) in 0.1 M PB, in case there was any glutaraldehyde remaining in the sample. The blood cells were incubated in 50 $\mu\text{Ci/ml}$ of ^3H - or 10 $\mu\text{Ci/ml}$ of ^{125}I -Concanavalin A (NEN, USA) in 0.1 M PB for 15 min at 37°C. The control blood cells were incubated in the same radioactivity of ^3H - or ^{125}I -Con A in 0.1 M PB containing 0.05 M α -methyl-d-mannoside for 15 min at 37°C. All labeled cells were postfixed with 2.5% glutaraldehyde in 0.1 M PB containing 1% tannic acid (7, 8, 22, 23, 24, 26, 27) for 30 min at 4°C, followed by 2% osmium tetroxide in the same PB for 30 min at 4°C after rinse. For electroconductive and for the stabilization (7, 30) of the surface structure, the cells were immersed in 1% tannic acid in distilled water for 30 min at 4°C, and then transferred into 1% osmium tetroxide in distilled water for 30 min at 4°C, followed by a rinse with distilled water. After dehydration with graded ethanol and substitution with 3-methylbutyl acetate, several drops of suspending cells were spread on a glass slide treated with 0.1% poly-L-lysine solution (43) for 10 min, and then dried in CO_2 with a critical point dryer (JCPD-3, JEOL). The measurement of radioactivity of exhausted solutions during the sampling procedure and the quantitative analysis of binding sites of Con A receptors with ^{125}I -Con A were done with a well type γ -ray counter (PACKARD-5260).

2) Autoradiography

The specimens on a rotary stage were coated by carbon at 45 degrees in a vacuum evaporator (JEOL-4C) with a cooling trap of liquid nitrogen. The degela-

tinized emulsion was prepared as follows: 1) Ilford-L4 or Sakura NR-H2 emulsion diluted 10 times with distilled water at 40°C was centrifuged at 5,000 rpm for 5 min at 40°C, 2) after removing the supernatant solution, the remainder of emulsion was further diluted with an equal volume of distilled water at 40°C. The specimens on a glass slide were dipped into the prepared emulsion and withdrawn with a slow and uniform motion (5). The excess emulsion was drained and the slides set to dry with the bottom up. The uniformity of the emulsion was checked with SEM. The exposure time was varied from 1 to 8 weeks in dark box under low humidity at 4°C.

Ilford-L4 emulsion coated samples were latensified with gold chloride (29, 37) for 15 to 30 sec at room temperature, briefly rinsed with distilled water at 15°C, and then developed with Phenidon developer (17, 29) for 60 sec at 10 to 15°C. Sakura NR-H2 emulsion coated samples were developed with Konidol-X for 4 min at 20°C. All samples were fixed with 24% sodium thiosulfate solution for 5 min and rinsed with distilled water. The gelatin was removed with distilled water at 45°C for 5 min. Then the specimens were dehydrated with graded ethanol and dried in CO₂ with a critical point dryer. After coating with platinum in an ion coater (Eiko IB-5), the specimens were observed under an SEM (Hitachi S-430) with the accelerating voltage at 25 KV.

RESULTS

The average diameter of silver halide crystals in the Ilford-L4 emulsion was estimated to be about 0.15 μm (28), and a monolayer of crystals covered the red blood cell surface uniformly and compactly, except in the concave region where the crystals were piled up somewhat (Fig. 1). The diameter of silver halide crystals in the Sakura NR-H2 emulsion was estimated to be about 0.1 μm (28), and a monolayer covered the erythrocyte homogeneously (Fig. 2).

³H- or ¹²⁵I-Con A binding sites on the surface of the red blood cells could be observed with the SEM-ARG method. The surface of the red blood cells was well preserved in spite of the autoradiographic procedure such as development and fixation (Figs. 3, 4). In an inhibition experiment to demonstrate the ³H- or ¹²⁵I-Con A binding sites with α -methyl-d-mannoside, only a few developed silver grains were scattered on the red cells and on the glass slide (Fig. 3): the average number was 1.48 ± 0.70 (³H-Con A-labeled control specimen) or 2.53 ± 0.82 (¹²⁵I-Con A-labeled control specimen) per single red blood cell face after exposure for 8 weeks.

The results of radioactive yield and quantitative analysis showed that the radioactivity of ¹²⁵I-Con A leaked from the samples into each waste solution during the sampling procedure (Fig. 5). The efflux of radioactivity into the osmium tetroxide fixative was highest, the value being 35% of all radioactive efflux. The radioactivity of ¹²⁵I-Con A remaining in the sample decreased to 38% after the substitution with 3-methylbutyl acetate, and the average radioactivity was 0.018 dpm per erythrocyte.

In the Phenidon developed specimen, the number of developed silver grains on the red blood cell surface increased in proportion to the length of exposure (Fig. 6). After exposure for 8 weeks, the average number of developed silver grains was 312.46 ± 76.07 (³H-Con A-labeled specimen) or 372.58 ± 106.05 (¹²⁵I-Con A-labeled specimen) per single red cell face. The SEM image of the developed silver grains, which was developed by the Phenidon method, had high contrast and the grains

FIG. 5. Efflux index of ^{125}I -Con A during sampling procedures after incubation. Heavy broken line connecting solid circles: efflux rates of erythrocyte samples, hatched bar: efflux rates into exhausted solutions, GA-TA: 2.5% glutaraldehyde fixative containing 1% tannic acid, Os: 2% osmium tetroxide fixative, TA: 1% tannic acid staining solution; Os*: 1% osmium tetroxide staining solution, 50-95: 50-95% graded ethanol, 100: 100% ethanol, 3-MA: 3-methylbutyl acetate.

appeared in spherical form (Fig. 7). Under high magnification, the grains were observed as polyhedrons with diameters ranging from 0.3 to 0.4 μm (Fig. 8). On the other hand, the silver grains resulting from development of Sakura NR-H2 emulsion by a conventional procedure with Konidol-X had a filamentous form (Fig. 9). It was easy to distinguish the developed silver grains on the red cell surface. The developed silver grains were distributed homogeneously on the cell surface in both developing methods. There was no difference in the distribution pattern, and the grains developed by the Phenidon method seemed superior for SEM observation, as the grains were compact polyhedrons, in contrast to the filamentous shape of conventionally developed silver grains. The size of the grains developed by the

FIG. 1. Scanning electron microgram of human erythrocyte after coating with diluted and degelatinized Ilford-L4 emulsion. Silver halide crystals cover the erythrocyte surface homogeneously. $\times 10,500$ (Bar, 2 μm)

FIG. 2. Scanning electron microgram of human erythrocyte after coating with diluted and degelatinized Sakura NR-H2 emulsion. Silver halide crystals uniformly cover the surface of the erythrocyte. $\times 10,500$ (Bar, 2 μm)

FIG. 3. Scanning electron microscope autoradiogram of human erythrocytes after incubation in ^3H -Con A containing α -methyl-d-mannoside, coating with Ilford-L4 emulsion, exposure for 8 weeks, and development with Phenidon for 60 sec at 15°C. A few developed silver grains are observed on the erythrocyte surface and on the glass slide (arrows). $\times 2,400$ (Bar, 10 μm)

FIG. 4. Scanning electron microscope autoradiogram of human erythrocyte after incubation in ^3H -Con A containing α -methyl-d-mannoside, coating with Ilford-L4 emulsion, exposure for 8 weeks, and development with Phenidon for 60 sec at 15°C. Several small protrusions are observed on the erythrocyte surface (arrows). $\times 11,800$ (Bar, 2 μm)

FIG. 6. Average number of developed silver grains per single erythrocyte face after various exposure time. a: specimen labeled with 50 µCi/ml of ³H-Con A (Ilford-L4 emulsion, Phenidon development), b: specimen labeled with 10 µCi/ml of ¹²⁵I-con A (Ilford-L4 emulsion, Phenidon development).

Phenidon method at 10°C was estimated to be 0.1 to 0.2 µm in diameter (Fig. 10). The distribution of the grains was generally homogeneous, but clusters of several developed silver grains were frequently observed. Background fog was negligible in all the experiments, as it was in the inhibition experiment using α -methyl-d-mannoside.

DISCUSSION

1) Emulsion Coating

Generally, autoradiography in biological specimens is performed on thin sections

FIG. 7. Scanning electron microscope autoradiogram after incubation in ³H-Con A, coating with Ilford-L4 emulsion, exposure for 6 weeks, and development with Phenidon for 60 sec at 15°C. High contrast, spherical silver grains are distributed homogeneously on the erythrocyte surface. $\times 5,400$ (Bar, 5 µm)

FIG. 8. A higher magnified scanning electron microscope autoradiogram after incubation in ³H-Con A, coating with Ilford-L4 emulsion, exposure for 8 weeks, and development with Phenidon for 60 sec at 15°C. The developed silver grains have a polyhedral structure with diameters from 0.3 to 0.4 µm. $\times 11,800$ (Bar, 2 µm)

FIG. 9. Scanning electron microscope autoradiogram after incubation in ³H-Con A, coating with Sakura NR-H2 emulsion, exposure for 8 weeks, and development with Konidol-X for 4 min at 20°C. Filamentous developed silver grains are distributed homogeneously on the erythrocyte surface. $\times 11,800$ (Bar, 2 µm)

FIG. 10. A higher magnified scanning electron microscope autoradiogram after incubation in ¹²⁵I-Con A, coating with Ilford-L4 emulsion, exposure for 8 weeks, and development with Phenidon for 60 sec at 10°C. The developed silver grains are estimated to be from 0.1 to 0.2 µm in diameter, and they are distributed homogeneously, but clusters of several grains are also observed here and there. $\times 21,000$ (Bar, 1 µm)

supported on a glass slide or a grid in both light and electron microscope ARG. For emulsion coating, dipping (5, 15, 19, 29), dropping (29, 37), wire loop (10, 29, 31), touching (9, 29) and stripping techniques (18, 29, 34, 44) have been used. However, difficulties arise when one tries to apply ARG in SEM observation, because the surfaces of specimens are generally irregular and uneven. As material for SEM-ARG, Paul *et al.* (33) chose cellulose fibers which have a comparatively flat surface. Hodges and Muir (11) reported SEM-ARG using paraffin sections. These experimental materials are very good because of the facility of emulsion coating.

Junger and Bachmann (14) checked the uniformity of the emulsion coating in several coating techniques under SEM. The wire loop technique was unsatisfactory for the three-dimensional cell surface, but a uniform, densely packed emulsion layer was obtained when the cells are coated with a stripping film. However, we think that although the stripping film make good contact with the cell top, it does not make contact with depressions in the irregular and uneven cell surface. Furthermore, in this technique the coating is liable to peel from the specimen and bubbles can appear between the specimen and the film (16), even though stripping film is very thick and contains a large amount of gelatin. Therefore, we think that this method is useless for SEM-ARG.

In the present study, human red blood cell specimens were coated with carbon in a clean vacuum condition after the sample was spread on the glass slide; they were then covered with diluted, degelatinized emulsion by a dipping method, and dried with the bottom up. The silver halide crystals of the Sakura NR-H2 emulsion were more evenly distributed than those of Ilford-L4. This difference may be due to the fact that silver halide crystals of the Sakura NR-H2 emulsion are smaller than the crystals of the Ilford-L4 emulsion.

2) Artifact Problems

The reinforcement and protection of specimens during the autoradiographic procedure is very important in SEM-ARG. If one uses a conventional fixation method with glutaraldehyde and osmium tetroxide for SEM-ARG specimens in our experiments, artifacts such as depressions and cracks in the red blood cell surface will appear during the autoradiographic procedure. In the report by Sudár and Csaba (40), the cell surface structures are well preserved in spite of the autoradiographic procedure. This is probably due to the protection afford by the deposit carbon and gold on the cell surface by evaporation under vacuum conditions before the emulsion coating. However, the thin gold film will absorb β -rays from the specimen and this may decrease the number of developed silver grains (25).

Tannic acid and osmium tetroxide for electroconductive staining are useful to preserve the cell surface structures. But this may not be always the method for preservation or fixation of radioactive isotopes in the specimen, because there is some efflux of ^{125}I -Con A into the exhausted solution, especially in osmium tetroxide solutions.

3) Removal of Gelatin

Generally, in TEM-ARG the removal of gelatin is done after the developmental procedure. In SEM-ARG, the removal of gelatin is required in order to observe fine cell surface structures, because the resolution and the contrast of the SEM image

are reduced remarkably by the gelatin layer remaining on the cell surface. In other words, it is necessary to use a low-gelatin emulsion for coating the specimen. Of course, the gelatin must be removed from the cell surface after development even when the emulsion has been degelatinized before the coating the specimen. In our case, the gelatin was removed with distilled water at 45°C after fixation. This was to protect the fine cell surface structures and also to prevent the dropping of developed silver grains from the cell surface.

4) Distribution of Developed Silver Grains

In our results, the developed silver grains were distributed homogeneously on the whole cell surface. After blocking Con A binding to the cell surface by α -methyl-d-mannoside, only a few grains were observed as a background fog level. The distribution of developed silver grains represents the Con A receptor sites, and is well consistent with the results of other Con A labeling methods (1, 12, 32, 36, 39). After exposure for 8 weeks, the average number of developed silver grains was 312.46 ± 76.07 (^3H -Con A-labeled specimen) or 372.58 ± 106.05 (^{125}I -Con A-labeled specimen) per single erythrocyte face. Calculating from these data, the average number of developed silver grains is about 7×10^2 per whole red blood cell. On the other hand, the number of Con A receptors in human red blood cells has been reported to be 1.2×10^5 (38) or 2.2×10^6 (35) in biochemical research. Moreover, the number of Con A receptors represented morphologically by several markers has been determined to be 1.2×10^4 (Con A-hemocyanin) (39), $6\text{--}10 \times 10^4$ (native Con A) (1), $1.2\text{--}2 \times 10^4$ (Con A-ferritin) (1), and 2×10^4 (Con A-colloidal gold) (12). Thus the number of receptor sites reported by other investigators is higher than that of our data. The discrepancy may be due to several peculiarities of ARG such as the size of silver halide crystals in the emulsion, efflux of Con A during the sampling procedure, radioactivity of the Con A used, the half time of the radioisotope selected, exposure time, and absorption of β -rays during repeated electroconductive treatment with tannic acid and osmium tetroxide. For example, assuming a surface area of $110 \mu\text{m}^2$ for one red blood cell (20), a compact monolayer of Ilford-L4 emulsion should contain about 5×10^3 of silver halide crystals ($0.15 \mu\text{m}$ in diameter) per whole erythrocyte. If all silver halide crystals are latensified by radioactive Con A, the maximum number of developed silver grains formed is no more than about 5×10^3 per red blood cell. This value is even lower than that of the Con A receptors reported by other investigators.

Although several problems remain, our study has shown that SEM-ARG is still very useful for the three-dimensional observation of receptor sites, including Con A receptors on the cell surface. We intend to try further work with smaller silver halide crystals in the emulsion, and with improved fixation methods for the radioactive compounds during the sampling procedure.

Caro (4) and Bachman and Salpeter (2) have published papers regarding resolution in ARG. Mizuhira and Futaesaku have described the theoretical problems of sensitization of silver halide crystals by β -rays using various kinds of nuclear sources and developmental conditions (25). Although these reports are very worthy to study resolution, they are descriptions of the resolution in TEM-ARG, and it seems that these interpretations are not necessarily able to apply to the examination resolution in SEM-ARG. The study of resolution in SEM-ARG would be examined

minutely in the future investigation.

In conclusion, a remarkable three-dimensional demonstration of the Con A receptor sites of human red blood corpuscles was made with an improved SEM-ARG method. This method is a practical and useful technique to demonstrate the binding sites of radioactive compounds, including receptor binding sites.

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