Application of a Backscattered Electron Image to Immunocytochemistry in Freeze-Cracked Tissues

Tatsuo USHIKI,1 Katsuhisa YONEHARA,2 Toshihiko IWANAGA and Tsuneo FUJITA1

Department of Anatomy (Prof. T. FUJITA),1 Niigata University School of Medicine, Niigata.
and Application Laboratory,2 Naka Works, Hitachi Ltd., Katsuta, Ibaraki, Japan

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Summary. A backscattered electron (BSE) image was applied to the observation of freeze-cracked tissue blocks stained with the indirect immunoperoxidase method. On the cracked surface of the pancreas, the BSE image clearly revealed the immunostained B cell granules as bright spots. A pair of secondary electron and BSE images on a fractured surface are useful for the three-dimensional localization of antigens in cells.

Recently, several investigators have demonstrated the utility of SEM in concert with backscattered electron (BSE) imaging for the visualization of cellular components beneath cell surfaces (ABRAHAM and DEENE, 1973; UN AND BECKER, 1975; DEENE and ABRAHAM, 1976; BECKER and SOGARD, 1979). Because of the atomic number contrast present in the BSE signal, cellular components stained with heavy metal are able to be visualized.

Diaminobenzidine, the product in enzyme (peroxidase) immunohistochemistry has a strong affinity to osmium. HARTMAN AND NAKANE (1981) proposed that enzyme-histochemical preparations, after osmification, are useful for ultrastructural localization of antigens by BSE imaging. The specimens used by these authors have been tissue sections mounted on glass slides. Their intention in the use of SEM has been to elevate the resolution in the localization of antigens.

The present study aims to show the applicability of these BSE images to the immunohistochemical localization of intra- and extracellular antigens in tissues with more massive and three-dimensional structures.

As the first step, this paper will demonstrate the intracellular localization of the peroxidase-labeled insulin in islet B cells exposed on the freeze-cracked, considerably uneven surface of the pancreas.

MATERIAL AND METHOD

Adult wistar rats (male, body weight 150-200 g) were deeply anesthetized with Nembutal and perfused through the heart with Ringer’s solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The pancreas was removed, cut in
pieces and immersed in the same fixative for 4 hrs at room temperature. The specimens were rinsed in water, immersed in 70% ethanol and freeze-cracked by the use of liquid nitrogen. They were hydrated and washed for several minutes in 0.1 M phosphate buffer. Then the blocks were treated for 4 hrs in 0.3% Triton X-100 in 0.01 M phosphate buffered saline and immunocytochemically stained with the indirect immunoperoxidase method by Nakane and Pierce (1966). The anti-insulin serum used in this study had been raised in a guinea pig. The blocks were incubated for 12 hrs at room temperature with the antiserum, at a dilution of 1:800. After rinsing in the buffered saline, the blocks were incubated with peroxidase-labeled goat anti-serum against guinea pig IgG (1:180) for 2 hrs. Control tissue preparations comprised: 1) specimens treated with diaminobenzidine, without receiving any antisera, and then osmificated; and 2) specimens treated with immunohistochemistry using an antiserum absorbed by the antigen and then osmificated. Following immunostaining, the specimens were immersed in 1% OsO₄ for 1 hr, dehydrated in a graded series of ethanol up to 100%, and critical point-dried in CO₂. The dried tissues were mounted on aluminum stubs with the carbon paste and coated with carbon by evaporation to a thickness of about 10 nm. The specimens were examined in a Hitachi S-530 SEM equipped with a GW type 30 backscattered electron detector, which is a large area annular solid state detector. The secondary electron (SE) imaging was performed at 10 kV and BSE imaging at 20 or 25 kV accelerating voltage and probe current $1 \times 10^{-11}$ to $10^{-7}$ A.

RESULT AND DISCUSSION

The fine structure of the fractured surface of the exocrine and endocrine pancreas could be clearly observed in the SE images of the present specimens (Fig. 1a). There was little damage on the fractured surface morphology which might have been caused by the immunostaining procedures. Some electron charging artifacts were often found because of the single coating with carbon.

The BSE image of the same area (Fig. 1b) visualized the immunostained parts of the tissue as highlights, which obviously correspond to the B cells of islets. The nuclei of the cells were negative in reaction. Observation at a higher magnification clearly revealed that the reactive or bright matter was granular in structure (Fig. 2). Erythrocytes and fat cells also appeared bright in contrast.

The BSE image contained information relating both to the atomic composition and the topographical feature of the sample. The former is based on the atomic number of the substances contained in the sample. The latter refers to the elevations and indentations of the fractured surface.

The BSE image in the present specimens was mainly due to the atomic number (Z) contrast, while the topographic contrast was weak, as shown in Figure 1c which illustrates the BSE image caused only by topographic factors. The atomic number contrast of the BSE image of the immunoreactive B cells is due to the heavy metal osmium (Z=76), which is deposited at the sites of the occurrence of diaminobenzidine (DAB) reaction due to peroxidase. The contrast in erythrocytes is caused by their widely known osmiophilia. Pietra et al. (1969) ascribed this nature to a "peroxidatic
Fig. 1. Legend on the opposite page.
activity of hemoglobin" of erythrocytes. The contrast shown by fat cells is due to the well-known osmiophilic nature of lipids. The control preparations (see Material and Method) showed no specific contrast in B cells, while erythrocytes and fat cells showed a strong contrast. Therefore, comparison with control preparations is necessary and useful to distinguish immunostained components from non-specifically stained elements. Fortunately, however, erythrocytes and fat cells are usually easy to identify due to their morphology and location.

In this study, we demonstrated that BSE imaging is useful for enzyme immuno-cytochemistry applied to fractured tissue blocks. Observation of stereo-pairs of BSE images increases the information of antigen localization, as BSE beams come from considerably deeper portions of the specimen as compared with SE beams. It is expected that the BSE imaging of immunohistochemistry can be used to determine the exact correlation of antigen location with the SE images of cells and sub-cellular structures.

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牛木 辰男
〒951 新潟市旭町通1
新潟大学医学部
第三解剖学教室

Dr. Tatsuo USHIKI
Department of Anatomy
Niigata University School of Medicine
Asahimachi, Niigata
951 Japan