Advantages of fixed stored materials for immunoelectron microscopy, with special reference to the study of malignant lymphomas

H AKATSUKA, K KITO, M SHAMOTO,* T SUCHI
Department of Pathology and Clinical Laboratories, Aichi Cancer Center Hospital, 81-1159, Kanokoden Tashiro-cho Chikusa-ku, Nagoya 464, Japan, and the
**Department of Pathology, Fujita-Gakuen, University School of Medicine, Toyoake, Aichi, Japan*

Recent developments in the production of monoclonal antibodies have helped considerably in understanding the human immune system and its abnormalities. The distinction of T cell subsets is important not only in studies of the functional characteristics of lymphocytes but also in the investigation of the neoplastic processes of lymphocytes.¹⁻⁴ Immunoelectron microscopic methods allow identification of lymphocytes by means of their ultrastructural characteristics.^{5,6} It is difficult, however, to achieve satisfactory preservation of morphology at the electron microscopic level while preserving antigenicity, and usually all procedures must be carried out successively and without delay after the specimens are taken. We report here a method by which both antigens and ultrastructural morphology can be preserved for at least one year after fixation.

Material and methods

Ten lymph node biopsies each from T and B cell lymphomas diagnosed by clinical, histological, and immunological examination and five non-neoplastic lymph nodes were used for this study.

FIXATION, EMBEDDING, AND STORAGE

Specimens were cut into slices less than 1 mm thick. They were fixed in a periodate-lysine-paraformaldehyde⁷ for 4-6 h at 4°C with continuous agitation. Then they were washed in two to three changes of cold phosphate buffered saline (PBS). The specimens were agitated gently at 4°C in PBS containing 10% sucrose overnight, 15% sucrose for 8 h, 20% sucrose again overnight, and 20% sucrose with 10% glycerin for 5 h. The tissues were then completely embedded in OCT compound (Miles Laboratories Inc), quickly snap frozen by liquid

nitrogen, and stored at -80°C until required. On the basis of specimens examined so far, we have been able to preserve tissue satisfactorily at -80°C for up to one year, but it may be possible to preserve specimens for several years. When specimens are to be sectioned, they should be cut as soon as possible after removal from the deep freeze.

SECTION CUTTING AND STAINING

Sections of 10 µm thickness were cut in a cryostat and mounted on albumin coated slides. The sections on the slides were air dried for at least 1 h and then dried in a desiccator at 4°C overnight to ensure adhesion. They could be preserved in the desiccator at 4°C for three months. Before the sections were used, the desiccator in which they were kept was brought to room temperature in order to prevent condensation on the slides.

After washing in two to three changes of cold PBS containing 10% sucrose, the sections were allowed to react with appropriate antibodies—namely, OKT-3, 4 (Ortho Pharmaceuticals Ltd), Leu 3A and Leu 4 (Becton Dickinson FACS Systems), or both; OKT-8, OKIa (Ortho Pharm Ltd); and B1 (Coulter Electronics Inc). For negative control sections normal mouse serum was substituted for each primary antibody.

A three step immunoperoxidase technique was applied, using peroxidase-antiperoxidase (PAP) complex (mouse) specifically made for this purpose in our laboratory: first, the sections were allowed to react with the above optimally diluted antibodies in the wet chamber at room temperature for 2 h. Then they were washed in two to three changes of cold PBS containing 10% sucrose for 1 h at 4°C and incubated with biotinylated horse antimouse IgG (Vector Laboratories Inc) for 2 h at room temperature. They were again washed overnight in cold PBS containing 10% sucrose at 4°C and then incubated with both avidin-biotinylated peroxidase complex (Vector Laboratories Inc) and PAP complex (mouse)^{8,9} for 4 h at room temperature in the wet chamber.

After incubation they were washed in cold PBS containing 10% sucrose for 1 h, fixed in 2% glutaraldehyde for 10 min at room temperature, and washed again in two to three changes of cold PBS for 30 min. After this they were incubated first for 30 min in Karnovsky's solution without hydrogen peroxide and then in complete Karnovsky's solution for 10 min at room temperature, before washing in two to three changes of cold PBS for 15 min. The slides were then postfixed in 2% OsO₄ for 1-2 h at room temperature.

After dehydration in graded series of alcohol at 5-10 min intervals, the sections were embedded in

Fig. 1 A case of adult T cell leukaemia, $OKT-3^{+}4^{+}8^{-}$. $OKT-4$ positive cells are seen. Stored for 12 months. Unstained. $\times 4400$.

Fig. 2 A case of T cell lymphoma, $OKT-3^{+}4^{-}8^{+}$. $OKT-8$ positive cells are seen. Stored for six months. Stained. $\times 8100$.

Fig. 3 Case of B cell lymphoma. $B-1$ positive cells are found. Stored for six months. Stained. $\times 4400$.

Fig. 4 Case of B cell lymphoma. Ia positive cells are found. Stored for six months. Stained. $\times 5200$.

Technical methods

epoxy resin, quickly inverting a resin filled gelatin capsule on the section surface. During the entire procedure described the section remained attached to the slide. Following polymerisation of the resin, detachment of the capsule from the glass slide was facilitated by heating it over a Bunsen flame. Ultrathin sections were cut with a diamond knife on an LKB Ultratome and were examined either unstained or stained with uranyl acetate and lead under a Jeolco 100 CX electron microscope.

Results and discussion

The cases studied included T cell malignancies which might have been related in their origin to phenotypically and functionally distinct T cell subsets—in other words, OKT-3⁺4⁺8⁻ and OKT-3⁺4⁻8⁺ lymphomas, which could be commonly associated with helper/inducer and suppressor/cytotoxic functions. B cell malignancies were also studied.

The specimen shown in Fig. 1 was obtained from a case of adult T cell leukaemia. The plasma membrane of lymphoid cells with convoluted and indented nuclei was positive with OKT-4 antibody but negative with OKT-8 antibody. The cells of the adult T cell leukaemia showed characteristic features previously described.¹⁰ The plasma membrane of neoplastic lymphoid cells was positive with OKT-8 antibody in a case of OKT-8 positive T cell lymphoma (Fig. 2). On the other hand, the plasma membrane of B cell lymphoma was positive with B1 antibody (Fig. 3). Several Ia positive cells were also seen in a B cell lymphoma (Fig. 4). The method was useful not only for ultrastructural comparisons of neoplastic T and B lymphoid cells but also for the better characterisation of the T cell subsets.

Satisfactory preservation of ultrastructural morphology cannot always be obtained in immunoelectron microscopic studies by conventional methods. Moreover, it is not possible, by conventional methods, to repeat the reaction procedures when a first trial has failed. The new method enables us to repeat the reaction or apply new antibodies as they become available; while sections are available from stock. The residues of specimens after cutting can also be stored again by rapid freezing in OCT compound as described.

The washing time in this method may be rather long. Since more than 5 h is given to each step, to permit the penetration of liquid into the entire specimen, some minor adjustment of time is possible. The procedures after freezing can be per-

formed at any time if specimens are fixed according to our methods. We have usually carried out procedures on several cases simultaneously. Some investigators^{11,12} also store the specimens for light microscopic studies at -70°C until use. These stored specimens cannot be used for electron microscopic studies, however, because ultrastructural morphology may be poor. The specimens illustrated here had been stored in the freezer at -80°C for 6–12 months.

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Requests for reprints to: Hiromichi Akatsuka MT, Department of Pathology and Clinical Laboratories, Aichi Cancer Center Hospital 81-1159, Kanokoden Tashiro-cho Chikusa-ku, Nagoya 464, Japan.