COMBINATION OF FIXATION USING PLP FIXATIVE AND EMBEDDING IN PARAFFIN BY THE AMeX METHOD IS USEFUL FOR HISTOCHEMICAL STUDIES IN ASSESSMENT OF IMMUNOTOXICITY

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ABSTRACT — To establish a method for processing lymphoid organs suited to morphological, immunohistochemical and enzyme histochemical analyses for assessment of immunotoxicity, we examined a combination of fixation with periodate-lysine-paraformaldehyde (PLP) fixative and embedding in paraffin by the AMeX method (PLP-AMeX method). Spleen and thymus removed from monkeys and rats were fixed in PLP fixative for 6 hours at 4°C. After fixation, specimens were processed and embedded in paraffin by the AMeX method. In hematoxylin and eosin-stained sections, tissue architecture was well preserved. In immunohistochemical staining, markers of T lymphocytes (CD3, CD4, CD8), B lymphocytes (monkey: CD20cy, rat: CD45RA) and macrophage (monkey; CD68, rat: ED-1) were well identified according to their specificities, although the staining intensity of CD8 in the monkey and CD4 in the rat were somewhat weaker in PLP-AMeX-prepared sections than in those frozen. In enzyme histochemical staining, alkaline phosphatase activity was well preserved in neutrophils. In toluidine blue- and Giemsa-stained sections, eosinophil granules and the metachromasia of granules in basophil/mast cells were clearly detectable. These findings suggest that the PLP-AMeX method is a powerful tool for assessment of immunotoxicity.

KEY WORDS: PLP fixation, AMeX method, Paraffin section, Immunotoxicity, Immunohistochemical staining, Enzyme histochemical staining

INTRODUCTION

Histopathological examination of major lymphoid organs such as spleen, thymus, bone marrow, lymph nodes and mucosa associated lymphoid tissue provides a reliable indication of the immunotoxic potential of a compound (Bloom et al., 1987; Schuurman et al., 1994; De Waal et al., 1995; Gopinath, 1996; The ICICIS Group Investigators, 1998). Lymphoid organs contain various types of cells, such as lymphocytes, macrophage lineage cells, eosinophils, basophils and epithelial cells. Lymphocytes forming the lymphoid organs include various lymphoid subtypes, some of which appear to be morphologically similar under routine histopathological examination. Phenotypical classification of the cells is usually achieved by histochemical staining for surface markers, specific enzymes and other intracellular products.

Immunohistochemical and enzyme histochemical analyses are powerful tools for assessment of a compound's immunotoxic potential. Some antigens and endogenous enzymes lose their immunoreactivity and enzyme activity during routine processing using formalin fixation and paraffin embedding. Therefore, frozen sections with or without fixation are frequently used in histochemical staining. However, preparation of frozen sections is technically more difficult and the quality of frozen sections is often too poor for detailed histologic and cytologic observations.

A new paraffin-embedding method (the AMeX method) (Sato et al., 1986, 1992) using cold acetone fixation, dehydration by acetone, clearing by methyl...
benzoate and xylene consecutively and embedding in ordinary paraffin is now available for immunohistochemical staining. The AMeX method, as reported, can preserve many antigens difficult to detect in routinely formalin-fixed paraffin-embedded tissue sections (Sato et al., 1986). However, acetone fixation results in far more marked tissue shrinkage when compared to formalin fixation (Noguchi et al., 1997).

In our previous study (Suzuki et al., 2000), immunoreactivity and enzyme activity were well preserved in paraffin-embedded lung sections prepared by fixation with periodate-lysine-paraformaldehyde (PLP) fixative and embedding in paraffin by the AMeX method (the PLP-AMeX method). In addition, we reported that the lung architecture was very well preserved, and detailed histopathological findings of lung lesions induced by bleomycin could be observed in sections prepared by the PLP-AMeX method (Suzuki et al., 2000). This suggests that the PLP-AMeX method is useful for morphological, immunohistochemical and enzyme histochemical analyses, and will be applicable to analysis of changes induced by various compounds in lymphoid organs.

In the present study, we examined the PLP-AMeX method in order to establish a new method for processing lymphoid organs suited to morphological, immunohistochemical and enzyme histochemical analyses as a basis for future assessment of a compound's immunotoxic potential. Routine histopathological examination is a useful approach which fits neatly into the tiered approach approved by various regulatory agencies of the United States, Europe and Japan (Harling, 1996). The first tier of the test battery implies screening for immunotoxicity included in the assessment of the toxicological profile of a compound. In the case of drug-development studies, rats, dogs and monkeys are usually used in routine toxicity testing required by regulatory agencies. Rats are widely used to test the effects of various compounds, and monkeys frequently used in toxicity testing of peptide/protein drugs. Rats and monkeys were thus subjected to this study, and the spleen and thymus evaluated as representative lymphoid organs.

**MATERIALS AND METHODS**

**Animals**

*Macaca fascicularis* monkeys and rats (Sprague-Dawley) were used. Monkeys were purchased from CSK Inc. (Nagano, Japan) and subjected to experiments at the age of 5 years. Rats were purchased from Japan SLC Inc. (Shizuoka, Japan) and subjected to experiments at the age of 8 weeks. The animals were housed in cages in an animal room maintained at a temperature of 24 ± 2°C and humidity of 55 ± 10%, with 14 to 16 air changes per hour and 14-hr light and 10-hr dark cycles. The animals were sacrificed by exsanguination under anesthesia. These animal experiments were approved by the Ethical Committee for Treatment of Laboratory Animals at Chugai Pharmaceutical Co., Ltd.

**Tissue preparation**

At necropsy, the spleen and thymus were removed from each animal. The tissues were processed and embedded in paraffin by the PLP-AMeX method (Suzuki et al., 2000) as follows.

The tissues were fixed in PLP fixative (containing 4% paraformaldehyde) (McLean and Nakane, 1974) for 6 hr at 4°C. After washing in phosphate-buffered saline (PBS; 0.01 M, pH 7.4) at 4°C, the specimens were dehydrated in acetone overnight at 4°C and for 2 hr at room temperature, cleared in methyl benzoate for 1 hr and in xylene for 1 hr, soaked in paraffin for 2 hr at 60°C, and then embedded in paraffin. The paraffin blocks prepared by the PLP-AMeX method were kept at 4°C.

Thin paraffin sections of the specimens were deparaffinized in xylene and acetone and stained immunohistochemically and enzyme histochemically.

For comparison, three different tissue preparation methods were used: PLP-fixed frozen method (PLP-frozen method), AMeX method (Sato et al., 1986) and routine methods of fixation in 10% neutral-buffered formalin and embedding in paraffin (NBF-paraffin method). Preservation of tissue architectures was examined for all tissue preparation methods. Comparisons in staining profiles of immunoreactivity and enzyme activity were done among sections prepared by the PLP-AMeX method, PLP-frozen method and NBF-paraffin method.

**General staining**

HE-stained sections were subjected to histological evaluation of tissue architecture. To detect basophils/mast cells and eosinophils, sections were stained with toluidine blue or Giemsa.

**Immunohistochemical staining**

In monkeys, antibodies against CD3 (marker antigens of T lymphocytes, polyclonal, Dako, Carpenteria, CA, USA), CD4 (marker antigens of helper T lymphocytes, clone: OPD4, Biomedica, CA, USA), CD8
(marker antigens of cytotoxic/suppressor T lymphocytes, clone: DK25, Dako), CD20cy (marker antigens of B lymphocytes, clone: L26, Dako), CD68 (marker antigens of macrophage, clone: PK1, Dako) were applied as the primary antibodies.

In rats, antibodies against CD3 (marker antigens of T lymphocytes, clone: G4.18, Pharmingen, San Diego, CA, USA), CD4 (marker antigens of helper T lymphocytes, clone: W3/25, Harlan Sera-Lab, England, UK), CD8 (marker antigens of cytotoxic/suppressor T lymphocytes, clone: OX-8, Pharmingen), CD45RA (marker antigens of B lymphocytes, clone: OX-33, Pharmingen) and ED-1 (marker antigens of rat monocyte-macrophage lineage, BMA Biomedicals Ltd., August, Switzerland) were used.

Immunohistochemical staining was performed according to the labeled streptavidin-biotin (LSAB) method with a Dako LSAB kit (Dako). To retrieve some antigens, sections were treated with microwave heating in 0.01 M citrate buffer (pH 6.0) at 98°C for 10 min in a microwave oven (H2800; Energy Beam Sciences, Agawam, MA, USA) with a power setting of 780W prior to immunohistochemical staining. To increase the stainability of CD8 in monkey and CD4 in rat, additional immunohistochemical staining was performed according to the tyramide signal amplification-avidin-biotin complex (TSA-ABC) method with a Dako catalyzed signal amplification system (Dako). The immunoreaction was visualized by peroxidase-diaminobenzidine reaction. The sections were finally counterstained with hematoxylin.

Enzyme histochemical staining

To identify neutrophils, ALP activity was detected with naphthol AS-Bi phosphate (Sigma Chemical Co., St. Louis, MO, USA) as the substrate and fast red violet LB salt (Sigma Chemical Co.) as the coupler (Watanabe and Fishman, 1964). The sections were counterstained with hematoxylin.

RESULTS

Tissue architecture and staining profiles of HE, toluidine blue and Giemsa

In both monkeys and rats, tissue architecture in sections of spleen and thymus prepared by the PLP-AMeX method was distinctly well preserved compared to that of PLP-frozen sections (Photo 1a and 1c). Differences in morphologic preservation of sections prepared by the PLP-AMeX method and NBF-paraffin method were negligible (Photo 1c and 1d). In sections prepared by the AMeX method, there was a slight difference in tissue shrinkage when compared to the other two paraffin-embedding methods (Photo 1b).

Staining profiles of HE were compared among sections prepared by the three different paraffin-embedded methods; PLP-AMeX method, AMeX method and NBF-paraffin method. Under a routine HE-staining process, balance of staining intensity did not differ in sections prepared by the PLP-AMeX method or NBF-paraffin method. On the other hand, the staining intensity of eosin was stronger in the AMeX-prepared sections than in the other types. Modification of the staining process was thus necessary to obtain appropriate results in the AMeX-prepared sections.

Next, staining profiles of toluidine blue or Giemsa were compared between the PLP-AMeX-prepared sections and NBF-paraffin-prepared sections. Toluidine blue reacted with PLP-AMeX-prepared sections in the same manner as it did with NBF-paraffin sections. Metachromasia of granules in basophil/mast cells was clearly detectable in sections prepared by the PLP-AMeX method (Photo 2a). In addition, there were no differences in stainability of Giemsa between the PLP-AMeX-prepared sections and NBF-paraffin-prepared sections. Eosinophil granules were stained satisfactorily with Giemsa and metachromasia of granules in basophil/mast cells was clearly detectable in PLP-AMeX-prepared sections (Photo 2b).

Immunohistochemical staining in monkeys

Sections prepared by the PLP-frozen method were stained satisfactorily with all antibodies used in this study by the LSAB method.

In the sections prepared by the PLP-AMeX method, CD3, CD4, CD20cy and CD68 were readily identified according to their specificities by the LSAB method (Photo 3). Staining profiles of these markers were then compared between the PLP-AMeX-prepared sections and PLP-frozen-prepared sections. The distribution and staining intensity of CD20cy and CD68 did not differ in the two types of sections. Distribution of CD3 and CD4 was similar to that of the PLP-frozen sections, although staining intensity was somewhat weaker in the PLP-AMeX-prepared sections than in the PLP-frozen-prepared sections. The effect of microwave heating applied to the PLP-AMeX-prepared sections on immunoreactivity of CD3 and CD4 was then examined. Stainability of CD3 and CD4 was increased by microwave heating. When results obtained by microwave heating were compared with
those of PLP-frozen-prepared sections, there were no differences in either distribution or intensity. Anti-CD8 antibody did not stain the antigens by the LSAB method even after microwave heating pretreatment. By the TAS-ABC method, CD8 was detectable in PLP-AMeX-prepared sections. Distribution of CD8 in these sections was similar to that in the PLP-frozen-prepared sections, although staining intensity was somewhat weaker in the PLP-AMeX-prepared sections.

In the NBF-paraffin sections, anti-CD3, CD4, CD8, CD20cy, and CD68 antibodies were not stained by the LSAB method. After microwave heating pretreatment, CD3, CD4, CD20cy, and CD68 were detectable by the LSAB method. However, CD8 was not detected even with microwave heating pretreatment or with the TAS-ABC method.

**Immunohistochemical staining in rats**

Sections prepared by the PLP-frozen method were satisfactorily stained with all antibodies used in this study by the LSAB method. In sections prepared by the PLP-AMeX method, CD3, CD8, CD45RA, and ED-1 were clearly detectable according to their specificities by the LSAB method (Photo 3). There were no differences in distribution or staining intensity of these markers between PLP-AMeX-prepared sections and PLP-frozen-prepared sections. On the other hand, anti-CD4 antibody failed to stain antigens in the PLP-AMeX-prepared sections with the LSAB method, even after microwave heating pretreatment. Consistent and specific immunostaining of CD4 was obtained in the PLP-AMeX-prepared sections with the TAS-ABC method, although staining
PLP-AMeX method in assessment of immunotoxicity.

Photo 2. Thymus (a) and spleen (b and c) sections of a monkey prepared by the PLP-AMeX method. (a): Metachromasia of granules in basophil/mast cells (arrows) is detectable in toluidine blue-stained section. (b): Granules of eosinophils (arrows) stained with Giemsa. (c): ALP activity (arrows) is noted in neutrophils. ×100.

intensity was somewhat weaker in PLP-AMeX-prepared sections than in PLP-frozen-prepared sections.

In the NBF-paraffin sections, CD8 and ED-1 were clearly detectable by the LSAB method. However, CD3, CD4 and CD45RA were not detected even with microwave heating pretreatment or with the TSA-ABC method.

Enzyme histochemical staining
In both monkeys and rats, ALP activity was very well preserved in sections prepared by the PLP-AMeX method, and was noted in the neutrophils (Photo 2c). There were no differences in enzyme activity between PLP-AMeX-prepared sections and PLP-frozen-prepared sections. On the other hand, enzyme activity was completely lost in NBF-paraffin sections.

DISCUSSION
Although standard histopathological examination is a powerful tool for assessment of immunotoxic potential, routine procedures often fail to differentiate lymphoid subpopulations. Therefore, immunohistochemical staining of frozen sections is widely used for phenotyping cells. However, the quality of frozen sections is often too poor for morphologic observations.

The authors recently reported that the PLP-AMeX method, combining fixation with PLP fixative and embedding in paraffin by the AMeX method, resulted in better preservation of many antigens that cannot be detected in routinely prepared paraffin sections (Suzuki et al., 2000). Moreover, we reported that the architecture was very well preserved, and detailed histopathological findings could be observed in sec-
Photo 3. Spleen sections of a monkey (a, b and c) and a rat (d, e and f) prepared by the PLP-AMeX method. (a and d): CD3-positive T lymphocytes are observed in the periarterial lymphoid sheath. (b and e): CD20cy- or CD45RA-positive B lymphocytes are found in the lymph follicle. (c and f): Macrophages stained with CD68 or ED-1 are observed in the red pulp and germinal center. ×840.
PLP-AMeX method in assessment of immunotoxicity.

To classify the subpopulation of T lymphocytes in the PLP-AMeX-processed sections, two different immunohistochemical staining methods were necessary: the LSAB method for staining CD4 in monkeys and CD8 in rats and the TSA-ABC method for staining CD8 in monkeys and CD4 in rats. The staining procedure of the TSA-LSAB method is complicated when compared with the LSAB method. When the immunohistochemical classifications of T lymphocyte subtypes are needed for assessment of immunotoxicity, it is preferable to use both the PLP-AMeX method and PLP-frozen method.

In immunohistochemical staining, pretreatment with heating and/or enzymatic digestion is frequently used to increase antigen staining potential. In NBF-paraffin sections, pretreatment by microwave heating was needed to detect CD3, CD4, CD20cy and CD68 in monkeys. In contrast, the PLP-AMeX-prepared sections reacted with almost all antibodies, including monkey-CD3, CD4, CD20cy and CD68, without pretreatment. Pretreatment with heating and/or enzymatic digestion, therefore, does not seem necessary to identify antigens in sections prepared by the PLP-AMeX method. On the other hand, microwave heating increased the stainability of several antigens in the PLP-AMeX-prepared sections. Therefore, pretreatment with microwave heating is worth trying in cases in which staining intensity is insufficient and/or immunohistochemical staining without pretreatment has failed to detect antigens.

In enzyme histochemical staining, ALP activity in neutrophils was readily detectable in sections prepared by the PLP-AMeX method. In contrast, enzyme activity was completely lost in NBF-paraffin sections. These findings indicate that endogenous enzyme activity in the PLP-AMeX-processed sections is definitely better than that in routinely formalin-fixed paraffin sections. We concluded that the PLP-AMeX method is suitable for enzyme histochemical analyses for phenotyping cells.

In this study, we did not analyze the effects of storage on immunoreactivity and enzyme activity in PLP-AMeX-processed tissues. However, it was reported that immunoreactivity was well preserved in AMeX-processed tissues at 4°C for up to 24 months (Sato et al., 1992). Therefore, we are certain that when blocks prepared by the PLP-AMeX method are kept at 4°C, neither immunoreactivity nor enzyme activity are affected by prolonged storage.

The immune system is a multicompartment system comprised of lymphoid organs located throughout the...
whole body. As a preliminary study, we examined the application of the PLP-AMeX method to perfused-fixation of the entire body in rats. Animals were perfused through the left ventricle with PLP fixative, and the tissues were then processed and embedded in paraffin by the AMeX method. Consequently, many antigens and endogenous enzymes were readily identified according to their specificities. Therefore, the PLP-AMeX method is also useful for whole body perfused-fixation, and will be applicable for detailed assessment of a compound's immunotoxic potential by pathological examination.

In conclusion, T lymphocytes, B lymphocytes and macrophages were clearly identified in PLP-AMeX-prepared sections by immunohistochemical staining. ALP activity in neutrophils was readily detectable by enzyme histochemical staining. Moreover, eosinophils and basophil/mast cells were clearly identified with toluidine blue or Giemsa staining. Therefore, the PLP-AMeX method, combining fixation using PLP fixative and embedding in paraffin by the AMeX method, is useful for morphological, immunohistochemical and enzyme histochemical analyses of lymphoid organs, and is also a powerful tool for immunotoxicity assessment.

REFERENCES


