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EXPERIMENTAL AND TOXICOLOGIC PATHOLOGY

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A new method of wet scanning electron microscopy for the analysis of myelination in EAE mouse model of multiple sclerosis

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Received 29 September 2005; accepted 22 November 2005

Abstract

Development of effective therapies for multiple sclerosis (MS) is dependent on the advancement of improved tools for evaluation of progression of this disease in animal models. We present a novel technique utilizing scanning electron microscopy (SEM) for imaging wet biological specimens thus enabling rapid and high-resolution imaging of myelin in mouse spinal cord (SC). We demonstrate the advantages of using the wet SEM technique to image myelin in a murine model of MS, experimental autoimmune encephalomyelitis (EAE) induced in the Biozzi (antibody-high) mouse, by sensitization with spinal cord homogenate (SCH) in adjuvant. Our studies show that the methodology allows easy identification of normal and pathological components with great clarity, which is then correlated with light microscopy (LM) and validated thereby. Furthermore, we demonstrate gold immunolabeling of specific epitopes. We conclude that the new technique provides a quick, accurate, and detailed structural evaluation of the SC that can be applied to advance the research of MS.

Keywords: Wet scanning electron microscopy; Myelin; Multiple sclerosis

Introduction

Multiple sclerosis (MS) is a chronic disease of the central nervous system (CNS), and characterized by sharply demarcated areas of demyelination and axon injury associated with inflammatory activity resulting in disability (Keegan and Noseworthy, 2002; Lassmann, 2004; Lucchinetti et al., 2001; O'Connor, 2002). The etiology of the disease is unknown, but evidence suggests that it is an autoimmune disease directed against CNS components, occurring in genetically susceptible individuals. Inaccessibility of the CNS limits pathological studies in humans and has thus driven the search for animal models of MS so as to investigate pathological mechanisms and test therapeutic strategies (Roder and Hickey, 1996; 't Hart and Amor, 2003). Due to the apparent autoimmune nature of MS, rodents, sensitized with CNS tissue or sensitized by transfer of autoreactive T cells, exhibit experimental autoimmune encephalomyelitis (EAE) with multifocal-demyelination pathology similar to that of MS. In addition to extensive

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doi:10.1016/j.etp.2005.11.001

degenerating and demyelinating axons, the disease episodes are characterized histopathologically by extensive immunoglobulin deposition and infiltration of astrocytes, macrophages, T cells (mainly CD4-positive), and some B lymphocytes into the CNS. Myelin can be imaged by light microscopy (LM) employing conventional stains such as hematoxylin and eosin (H&E) and luxol fast blue or a variety of immunohistochemical procedures.

Recently we described a new technique for scanning electron microscopy (SEM) of wet tissue samples (WETSEM^{TM}) (Barshack et al., 2004a, b; Nyska et al., 2004; Thiberge et al., 2004a, b). The wet tissue samples are placed in sealed capsules, thus protecting the samples from the vacuum environment in the microscope chamber by a thin, electron-transparent membrane. High-resolution images can be obtained from specimens several millimeters thick without embedding and sectioning, thereby enabling rapid sample analysis. This is achieved due to the reflective mode of backscattered electron detection which enables use of unsectioned, fully hydrated specimens, thereby obviating thin tissue sectioning. The objective of this work was to evaluate and validate the wet SEM technique. The system that we used for our studies was an EAE-induced Biozzi AB/H (antibody-high) mouse by sensitization with spinal cord homogenate (SCH) in adjuvant, a model found to be useful for researching the chronic relapsing remitting (RR) characteristics of MS (Baker et al., 1990).

Materials and methods

Chemicals and solutions

Paraformaldehyde, glutaraldehyde, and uranyl acetate were purchased from Electron Microscopy Sciences (Hatfield, PA, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). McDowell's and Trump's (1976) 4F:1G (4 formaldehyde:1 glutaraldehyde) fixative (100 ml) was prepared by sequentially mixing the following by stirring: 71 ml distilled water, 25 ml 16% paraformaldehyde (EM grade), 4 ml 25% glutaraldehyde (EM grade), 1.16 g $NaH_2PO_4 \cdot H_2O$, and approximately 0.27 g NaOH; the pH was adjusted to 7.2–7.4 with 1 N NaOH. The flushing solution was made of 1 ml heparin solution (1000 units/ml), 1 ml sodium nitrite, and 8.5 g sodium chloride in 1000 ml deionized water. Uranyl acetate, pH 3.5, was made of 5% (w/v) stock solution in water, kept in the dark at 4 °C, and diluted in water immediately prior to use.

EAE induction and clinical scoring

The RR EAE disease appears clinically in Biozzi female mice between 15 and 20 days following immunization. EAE was induced in fourteen 7–9-week-old Biozzi female mice. On day zero the first inoculation with SCH was administered by subcutaneous injection of SCH in complete Freund's adjuvant enriched with *Mycobacterium tuberculosis* into one flank, followed by an identical booster injection a week later in the second flank (Baker et al., 1990). Five healthy mice (no EAE) of the same strain and age were used as a control group. All procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Research Council (Institute of Laboratory Animal Resources, 1996). The mice were examined and scored clinically from 15 days after EAE induction until the termination of the study at 60 days after initiation of treatment. All mice having a score of 5 were sacrificed for humaneness. All mice having scores of 1 and above were considered sick (Baker et al., 1990).

Perfusion, necropsy, and tissue handling

On day 60, mice were deeply anesthetized with isoflurane and air and transcardially perfused. The mice were initially perfused with a flushing solution until the perfusate was bloodless, thereafter the mice were perfused with the fixative (Dykstra et al., 2002; McDowell and Trump, 1976). The vertebral column was isolated by cutting away as much muscle as possible, sectioned distally at the last rib, and immersed in the fixative. The specimens were kept in the fixative for 2 days before the spinal cord (SC) was separated from the vertebrae. For LM, 2-mm thick slices, one from the thorax and the other from the cervix, were cut, embedded in paraffin, sectioned at 5–6 μm, stained with H&E, and imaged using an Olympus BX51 microscope. For wet SEM technique, adjacent sections of 300-μm thick, from the thorax and the cervix regions, were cut with an Oscillating Tissue Slicer (EMS, Hatfield, PA) and processed: slices were rinsed in water, treated with 1% (w/v in water) tannic acid for 5 min, rinsed in water, stained with 0.1% uranyl acetate for 10 min, and finally washed several times with water. Complete transversal sections of the SC were inserted into the specimen capsule of the wet SEM (QuantomiX, Ltd., Nes Ziona, Israel) with the surface to be imaged in close contact with the electron-transparent membrane. The sealed capsule was then placed on the specimen stage of the microscope which was subsequently evacuated (Nyska et al., 2004).

Scoring of histopathological changes

For the wet SEM technique, the complete cross-section of the SC was captured at low resolution (× 40), identifying the dorsal, lateral, right and left, and ventral

white matter columns. The SC was then imaged at higher magnifications (× 400– × 3200) in different regions of the white matter columns. Histopathological assessment of SC sections was performed in a blinded manner on sets of photos prepared for each animal. The relative number of axons having myelin-ring structures with irregular thickness and fragmentation or loss of myelin was scored in each image using an arbitrary scale ranging from 0 (no change) to 4, looking at × 400 photos. Consequently, for each animal, an assessment of the overall grade of myelin damage was expressed representing the highest score seen in any of the SC images. Similarly, LM slides were evaluated, using similar scoring considerations.

Gold immunolabeling

Tissue sections of SC of 300-μm thickness were gold-immunolabeled for imaging by the wet SEM technique. All incubations were performed at room temperature (RT) unless otherwise stated. Tissue specimens were washed 3 times, each for 5 min, in 1% BSA/PBS pH 8.2; permeabilized using 60 μl/ml Auto/Zyme (Biomeda, Foster City, CA, USA) for 6 min at 37 °C; washed again 3 times, each for 5 min, in 1% BSA/PBS, pH 8.2; blocked with 5% normal goat serum (NGS) (Aurion, Wageningen, The Netherlands) in BSA/PBS for 30 min; and subsequently incubated in the first antibody. Axonal labeling was performed with antineurofilament antibody (Chemicon, Temecula, CA, USA) diluted at 1:500 in green buffer (Zymed, San Francisco, CA, USA) for 2 h. After washing 3 times, each for 5 min, with 1% BSA/PBS + 0.05% Tween 20, pH 8.2, the secondary antibody [40-nm gold-conjugated goat antimouse (British Biocell International, Cardiff, UK), at a dilution ratio of 1:10 in 1% BSA/PBS, pH 8.2] was applied overnight at 4 °C. Finally, a series of washes, 5 min each, was carried out: once with 1% BSA/PBS + 0.05% Tween 20, pH 8.2; twice with 1% BSA/PBS pH 8.2; and 4 times with doubly distilled water. Myelin labeling involved a similar protocol, with antimyelin basic protein antibody (Chemicon, Temecula, CA, USA) at a dilution ratio of 1:500. In this case, the secondary antibody [2-nm gold-conjugated goat antimouse (British Biocell International, Cardiff, UK), at a dilution ratio of 1:200 in 1% BSA/PBS, pH 8.2] was applied for 2 h. Washes were similar to those described above, followed by 3 washes with doubly distilled water and, then, silver enhancement for 1.5 h (Aurion, Wageningen, The Netherlands).

Results

To evaluate and validate the new wet SEM technique, we compared adjacent SC sections by LM and wet SEM. Fig. 1 demonstrates a representative set of samples from mice exhibiting different degrees of myelin damage, comparing SC sections prepared for LM or wet SEM, using the same magnification (× 400). This figure illustrates: no change (grade 0 – healthy mouse), mild (grade 2), moderate (grade 3), or severe (grade 4) axonal and inflammatory changes. In uranyl acetate-stained SC sections imaged by wet SEM, the intact myelin structures appear as sharp dark rings, while in H&E-stained LM sections the myelin sheaths and axons appear as relatively homogeneous pale reddish-stained tissue. In SCs from sick mice, gradual glial-cell infiltration occurs in the white matter, and the myelin is reduced in amount or degraded in several places.

Higher-resolution images using the wet SEM technique can be obtained, as seen in Fig. 2. A wide range of magnifications is shown from a control and a severely sick mouse (grade 4), providing an overview of the whole tissue section prior to focusing on any preferred region, to provide high-resolution SEM micrographs. The dark myelinated structures are highlighted, as well as additional histological features, such as axons, oligodendrocytes, and inflammatory response.

To demonstrate an additional capability of the new technique, we performed a useful measure of protein expression in tissues by immunolabeling. This can be obtained for wet SEM using approaches similar to immunofluorescence or immunohistochemistry routinely used for LM hisotopathology. Fig. 3 shows two examples of epitope labeling in SC sections: labeling of axonal neurofilaments (Fig. 3A–C) and labeling of myelin by myelin basic protein (Fig. 3D–F). This methodology can be further useful to evaluate axonal degeneration or regeneration, a potentially valuable tool for assessing intervention in axonal degeneration.

Discussion

We demonstrated that the wet SEM technique enables imaging of fully wet tissue samples in a SEM, which yields high resolution, and simple and rapid processing of tissue samples. Furthermore, this study reveals that myelin, due to its high lipid content, is resolvable by wet SEM with remarkable clarity in a manner previously unfeasible without extensive labor. The new technique achieves a wide range of magnifications, from low to high, allowing imaging the whole tissue section and thereafter, focusing on any preferred region. Use of high-quality SEM images at low magnification facilitated the screening of relatively large portions of the total area in the white matter, while higher magnification imaging was useful in ascertaining tissue architecture at a high resolution. Thus, the wet SEM method is a new approach which bridges between light and electron

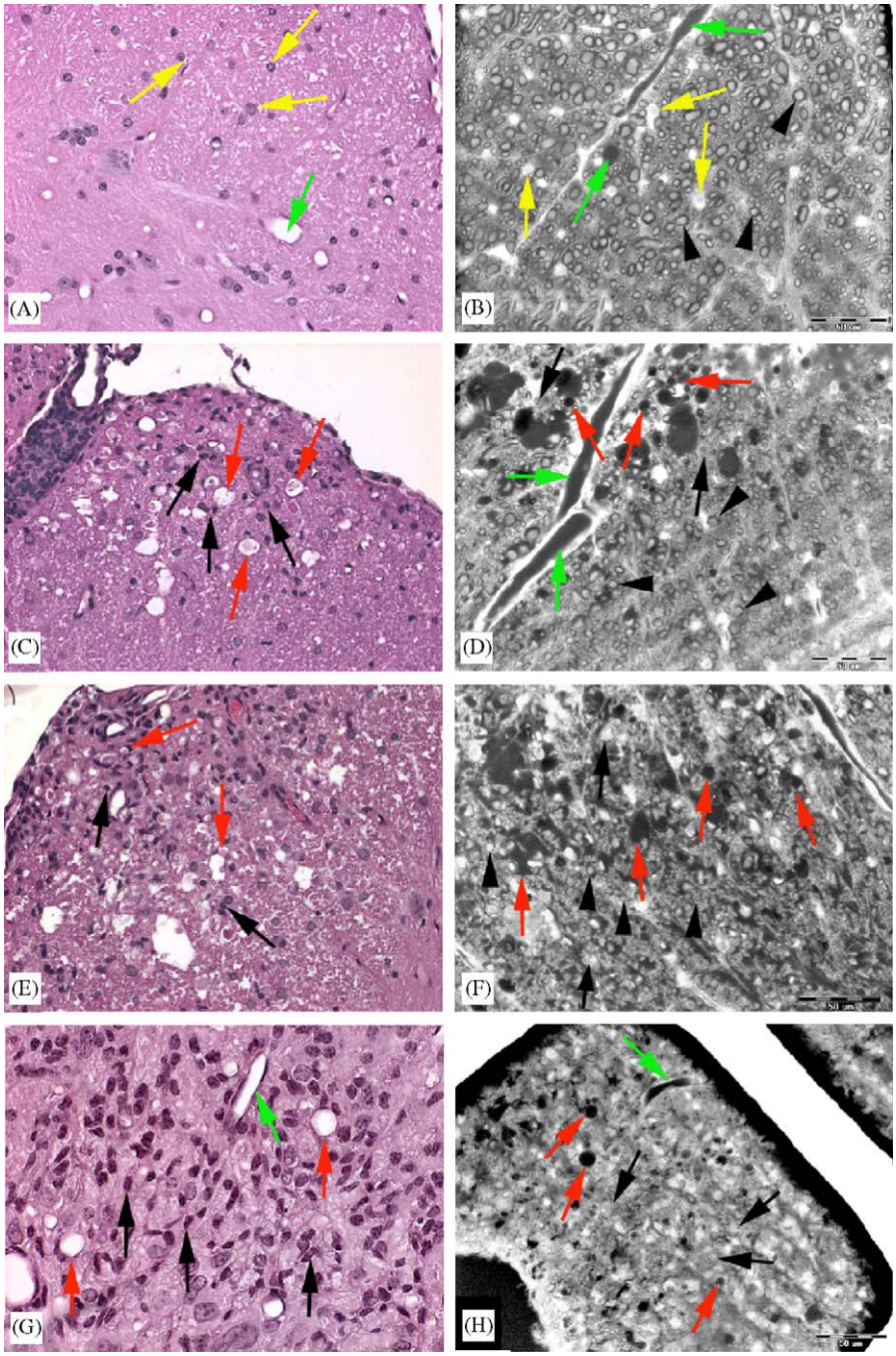
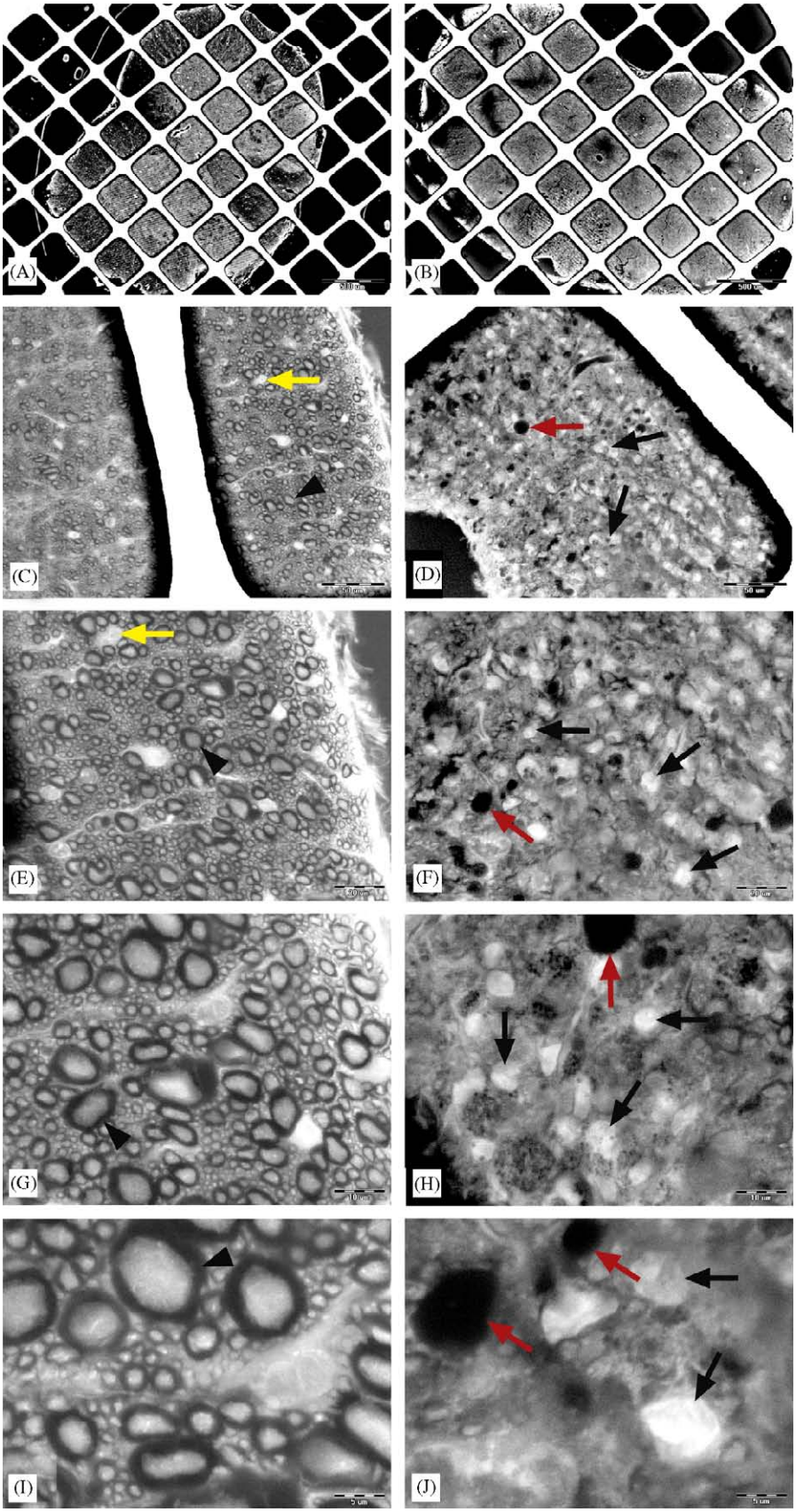


Fig. 1. Cervical spinal cord sections imaged either by LM or wet SEM methodology. Left panels: LM H&E-stained sections; right panels: wet SEM uranyl-stained sections; all at $\times 400$. (A, B) Normal mouse; (C, D) mild (grade 2); (E, F) moderate (grade 3); (G, H) severe (grade 4) white-matter damage. Same indicators used in both LM and wet SEM panels to show similar structures: green arrows – vascular spaces (irregular, dark, elongated spaces encircled by thin pale wall in wet SEM images), yellow arrows – oligodendrocytes (randomly distributed, round whitish features with grayish focus in middle), black arrowhead – myelinated axons [dark rings (myelin) with pale spaces (axons) in middle], red arrows – axonal and/or myelin loss (irregular, dark spaces), black arrows – microglial cells (single or multiple, irregular round whitish spaces with grayish focus in middle).

Fig. 2. Uranyl acetate-stained spinal cord section of normal and severely affected Biozzi mice using the wet SEM technique. Note that, at lowest magnification, "butterfly" shape of gray matter is seen gray. Same indicators are used in both the spinal cord photos from healthy ("normal") (left side photos), and in the spinal cord from the severely affected Biozzi mouse (right side photos). Yellow arrows – oligodendrocytes; black arrowhead – myelinated axons; red arrows – axonal and/or myelin loss; black arrows – microglial cells. (A, B) $\times 40$; (C, D) $\times 400$; (E, F) $\times 800$; (G, H) $\times 1600$; (I, J) $\times 3200$.


(A)
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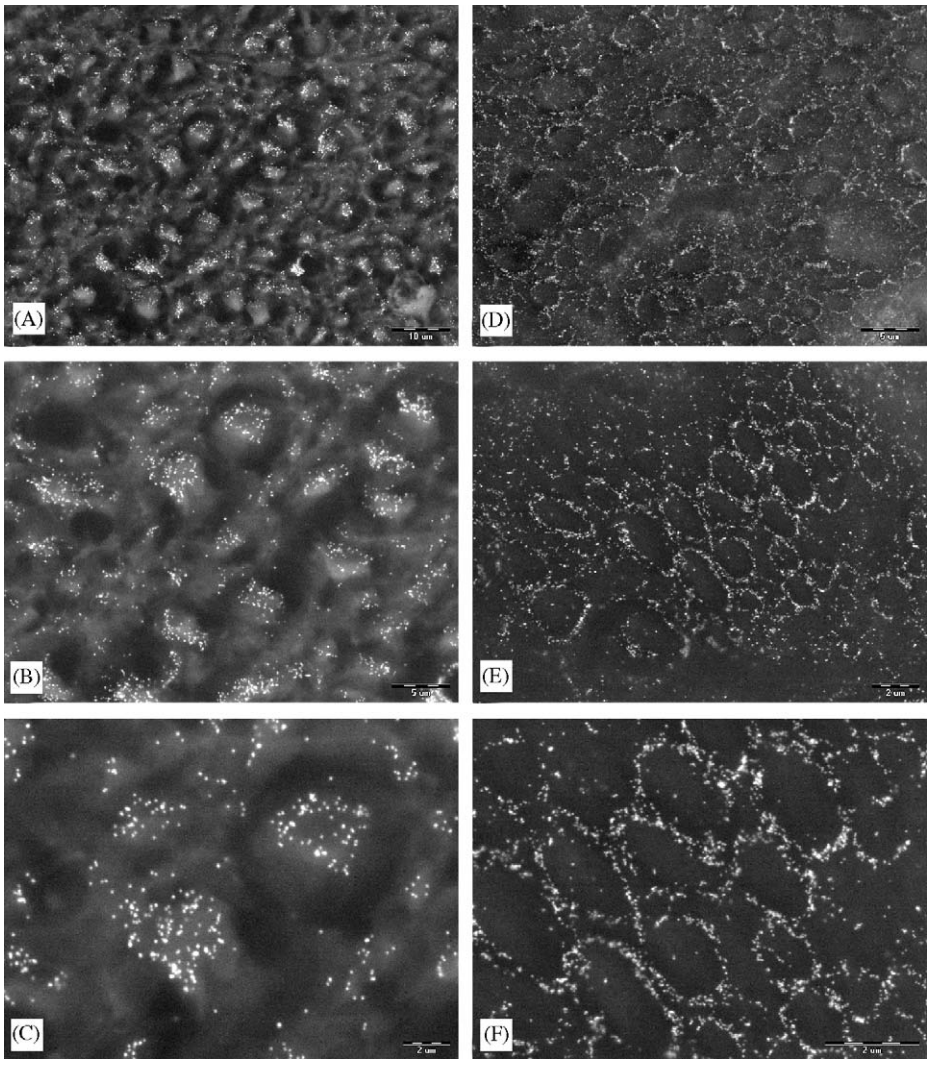

Fig. 3. Gold-immunolabeled spinal cord sections imaged by wet SEM technique at different magnifications. (A–C) Axonal labeling with antineurofilament antibody, (D–F) myelin labeling with antimyelin basic protein antibody. (A) $\times 1600$, (B) $\times 3200$, (C) $\times 6400$, (D) $\times 3200$, (E) $\times 6400$, (F) $\times 12\,800$.

microscopy in terms of range of achievable resolutions and straightforward sample handling procedures. Moreover, it enables visualization of wet tissues, thus minimal processing maintains the biological sample proximal to its original, native state.

In this work, the new wet SEM methodology was validated in a mouse model of MS by comparison with LM. For this purpose, $\times 400$ magnification was sufficient for a pathological scoring assessment, as the contrast achievable clearly reveals tissue ultrastructure. The advantage of using relatively low magnification for pathological evaluation is that it enables fast and clear screening of the tissue section. Where needed, however, high-resolution imaging is obtainable. In addition to tissue ultrastructure, we demonstrated that tissue gold-immunolabeling can result in high-resolution epitope localization. The procedure is similar to other immunolabeling used for LM. There are two main differences between immunolabeling for LM and wet SEM: (a) The use of wet tissue samples in wet SEM; and (b) High-resolution imaging of gold particles in the context of the tissue enables accurate localization of the labeled antigens.

We chose to test the potential application of the wet SEM technology in the MS model for two reasons. The search for effective new therapies for chronic degenerative and inflammatory disorders of the central and peripheral nervous system, such as MS, has been of particular interest to the pharmaceutical industry due to increased longevity and the associated higher frequency of these disorders in the general population (O'Connor, 2002). Secondly, great need exists for a variety of morphological screening methods beyond the routinely applied neurobehavioral analysis. We conclude that the wet SEM technique offers a novel, accurate assessment of myelination, a parameter likely to be invaluable in

assessing the efficacy of potential therapies for MS. We propose that the quality of the imaging of myelin generated by wet SEM will encourage more detailed investigations and provide new insights into the progression of MS.

Acknowledgments

We thank Niva Russek and Anya Vainshtein for their invaluable assistance in preparing the manuscript and Dr. Ayelet Chajut for critical reading and discussions.

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