Soft X-Ray Microscopy: A Tool for the Study of Multicellular Organisms

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INTRODUCTION

Electron microscopy is routinely used to study protozoa and small multicellular organisms since conventional light microscopy is inadequate to elucidate their structure. However, each electron microscopy technique has its inherent limitations. Scanning electron microscopy can reveal superficial characteristics of organisms and surfaces of tissue sections. Transmission electron microscopy can provide a wealth of morphological detail, but numerous sections are needed to depict the continuity and interrelationships of internal organs or structures. Confocal microscopy requires fluorescent label to identify the structure of interest. Recent applications[1-4] of soft X-ray microscopy to study unicellular organisms and the intracellular changes induced by *Plasmodium falciparum* in infected red blood cells yielded results which could not be obtained by other microscopy techniques, suggesting that this techniue may have wider applications in biosciences. This study was therefore undertaken to

1). - determine whether soft X-ray microscopy could be also be used to elucidate the structure of small multicellular organisms to complement the data obtained by other electron microscopy techniques, and

2). - to test the efficacy of selected vital stains to increase the carbon content of biological membranes, thus enhancing the contrast of images obtained.

The results presented are focused on the microfilaratiae of *Dirofilaria immitis*, the dog heartworm, and on the newborn larvae of *Trichinella spiralis,* a nematode parasite of vertebrates, which is acquired by eating raw or inadequately cooked meat (pork, bear) that contains the encysted, infective larvae. After being ingested, these larvae develop in the upper small intestine. Upon maturation and mating, the females produce newborn larvae which gain access to the circulatory system, become disseminated throughout the body, and infect voluntary muscle cells where they develop to the infective, encysted stage [5].

MATERIALS AND METHODS

The newborn larvae are approximately 7 x 110 μm in size They were obtained from in vitro cultures where *T. spiralis* adults, isolated from rats on the sixth day after experimental infection, were maintained. Microfilaraie of *D. immitis*, approximately 6 x 310 μm in size, were isolated from the blood of a dog which harbored a naturally acquired infection. All larvae were fixed in 4% glutaradehyde (Millonig's buffer, pH 7.5); some of the fixed larvae were processed for examination by transmission (Philips 201) and scanning (ETEC) electron microscopy[6]; others were examined by soft X-ray (MX-1) microscopy at the Advanced Light Source of the E. O. Lawrence Berkeley National Laboratory unstained, or after staining with either Methylene Blue, Azure II Blue, Trypan Blue, Crystal Violet or Lugols's Iodine.

RESULTS

The montages prepared from serial X-ray micrographs elucidated the internal structure of both larvae were too large for reproduction in this publication. The following organ systems or structures to be discerned by soft X-ray microscopy within the *T. spiralis* newborn larva body wall, the buccal area with stylet, nerve ring esophageal thread, stichosome, primordia of the intestine and gonads, rectum and structures hitherto undetected by other techniques, e.g. vaginal primordium (?); nerve cells in the anterior part of the larva which send axonal projections to the cephalic sensory structures, and a ganglion-like structure located in midbody region of the larva.

Transmission electron micrographs were correlated with the montage to elucidate finer morphological details at specific levels of the larva. Longitudinal and oblique sections through the cephalic space reveal a stylet in the buccal capsule and numerous axons converging onto the tip of the cephalic space. An axon bundle is present near the nerve ring. Section through the anterior portion of the stichosome shows prominent triradiate lumen of the esophagus surrounded by the stichocytes. Cytoplasmic granules are prominent within the stichocytes. Sections through the caudal region of the larva show undifferentiated primordia of the intestineand of the gonads, ciliary nerve endings of the caudal sensory organs and the cuticular lining of the rectum.

Three of the five stains tested enhanced the density of structures or components within the larvae. Methylene Blue stained dense material located in the apertures of the anterior sensory organs in *T. spiralis* larvae. Azure II Blue delineated the muscle bands, and Crystal Violet the nucleiin *D. immitis* microfilariae.

DISCUSSION

The combination of microscopy techniques used in this study allowed the elucidation of fine structure of both larvae, although the main emphasis was on elucidation of the stuctureof *T. spiralis* newborn larva. Data about *D. immitis* microfilaria will be presented in a separate report.

Scanning electron microscopy disclosed that the surface of the newborn larva is similar to that of the adult worms. Despite its small size, the larva has a fully formed body wall consisting of cuticle, muscle cells and hypodermis, and primorida of internal organ systems including the stichosome, intestine, and gonads. Cephalic space contains numerous sense organs which are cilia-like terminations of axons originating from cells located in the anterior portion of the larva. These presumed sensory receptors may function in assisting the larva to locate skeletal muscles. Similar cilia present near the posterior end of the larva, suggest the presence of caudal sensory organs.

A prominent stylet, located in the cuticularized buccal cavity, may be used by the larva to perforate sarcolemma during infection of skeletal muscle cells. Triradiate esophagus, extending from the buccal cavity to the intestine, is enveloped by the primordial stichosome; many stichocytes contain cytoplasmic granules, suggesting that the stichosome may already be functional, secreting products into the esophagus. The granules within the stichocytes, and the granular material within nerve cells in the anterior part of the larva, are autofluorescent and readily detected by confocal microscopy. Intestine appears to be non-patent. Length of the rectum may be used as a characteristic to determine the gender of the larva.

Whereas transmission electron microscopy provided a wealth of morphological detail, soft X-ray microscopy provided an excellent overview of the general structure of the larva, and was the key technique which depicted the location and interrelationship of its internal organs. Such overview is not possible to obtain by scanning nor by transmission electron microscopy. For example, the soft X-ray images strongly suggest that the space between the nerve ring and the cepahalic space contains nerve cells, many of which apparently innervate the cephalic sensory structures. In addition, this technique has allowed detection of structures which were hitherto either unknown or were not expected to be present at this stage of development, e.g. the gangion-like structure located in the mid-body region of the larva, and the lateral thickening of the inner body wall, resembling the vaginal primordium which is more defined in the mature muscle larvae.

These preliminary studies have demonstrated that the unique capabilities of soft X-ray microscopy can be successfully applied to examine the structure of small multicellular specimens, and indicated the potential use of this technology in

biological and biomedical sciences. In addition to providing new information about organization and interrelationship of internal organs, the images obtained by soft X-rays can serve as a valuable reference for interpretation of results obtained by transmission electron microscopy.

Although the images of unstained larvae were very informative, the internal organization of the specimens examined would be depicted more clearly if the contrast of the image could be enhanced. An initial test of five stains, all of which bind to cell membranes and proteinaceous structures, was made to determine whether the increasing the carbon content of the membranes by staining would enhance the contrast of the image obtained. The results suggest that three of the five stains tested, Methylene Blue, Azure II Blue and Crystal Violet, may be useful for contast enhancement. Methylene Blue rendered visible the dense material, probably glycoprotein in nature, located in the apertures of the anterior sensory organs. Azure II Blue rendered muscle bands of the microfilaria more apparent, and Crystal Violet appeared to have an affinity for the nucleus. Further studies are planned to determine which stain concentration:staining time combination will provide the optimum image contrast.

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