Chapter 5
Histologic Detection of Past Pathogens

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Abstract In recent years, histologic methods have been employed for the detection of infectious past pathogens (viruses, bacteria, parasites, or fungi) in ancient tissues. The goal of these palaeopathological studies is to further our understanding of the origin and spread of infectious diseases. Microorganisms can be visualised in preserved ancient tissues after different mummification processes, or in tissue sections from old paraffin blocks. The first step is the examination of paraffin sections with routine staining. Because organisms are often difficult to see in tissue sections, several special stains have been developed to visualise them. However, these histological stains are not specific. Electron microscopy may allow the detection of very small organisms such as viruses; the accurate identification of organisms is in some cases based on a specific morphology at the ultrastructural level. Depending on tissue storage conditions, immunohistological methods such as immunohistochemistry and immunofluorescence allow specific detection of microorganisms if antigenic epitopes are well preserved.

5.1 Introduction

The first step in the diagnosis of any infectious disease from recent or ancient tissue specimens is examination of tissue sections stained with hematoxylin and eosin (H&E). This histologic examination allows recognition of specific tissue and cytopathic changes, as well as consistent patterns of inflammation, and detection of microorganisms in H&E-stained sections. However, detection of microorganisms often poses a challenge for the histopathologist. Some microorganisms are too small to be seen easily by light microscopy, while larger-sized organisms may not be clearly distinguishable on H&E-stained sections because

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they are obscured by surrounding tissue elements. For these reasons, numerous special stains have been developed to detect organisms in paraffin sections. The stainability of a microorganism by a particular method does not mean that the organism can be identified accurately because many other organisms may show the same staining reaction. However, application of several judiciously chosen specific stains can allow a skilled observer to make a rapid preliminary identification of many organisms based on their morphology (Woods and Walker 1996). In practise, six special stains can be used to detect microorganisms in paraffin sections: Giemsa, Gram, periodic acid-Schiff (PAS), Grocott-Gomori methenamine silver (GMS), Warthin-Starry, and Ziehl-Neelsen stains (Lepidi et al. 2002a). The Giemsa stain is the most sensitive, allowing researchers to detect most microorganisms and study their morphology. The most popular and widely used histologic method for detection of bacteria is the tissue Gram stain. To a certain extent the Gram stain aids further identification of the organism because bacteria can be classified according to their Gram stainability as Gram-positive or Gram-negative bacteria. GMS and PAS stains detect most fungi. The Warthin-Starry stain is among the most sensitive methods for detection of microorganisms but is difficult to interpret (Lepidi et al. 2000). The Ziehl-Neelsen stain is used for detection of acid-fast bacteria, especially mycobacteria. The pattern of organisation should also be considered – for example, staphylococci and streptococci tend to gather together in clusters and in chains, respectively.

Electron microscopy can be useful in recognising and identifying microorganisms (Yardley and Hendrix 1961). Although interpretation may be somewhat hindered by suboptimal tissue preservation, examination by transmission electron microscopy may reveal the identity of a microorganism based on its specific morphology at the ultrastructural level. This morphological method has been employed in ancient tissues, e.g. in the identification of *Variola* virus in old formalin-fixed tissues and in an Italian mummy from the sixteenth century, or in identification of treponemes in a renaissance Italian mummy with syphilis (Fornaciari and Marchetti 1986; Fornaciari et al. 1989; Schoepp et al. 2004).

In the 1980s, immunohistology revolutionised histopathology, particularly with regard to the categorisation of solid tumours and haematopoietic neoplasms. Immunohistological methods are based on detection of antigenic determinants in tissue sections. These techniques mainly encompass immunohistochemistry and immunofluorescence. For several years, immunohistology has also been used for the identification of infectious agents. After histochemical staining, immunohistology is the most commonly used ancillary diagnostic technique for the detection of microorganisms in histologic sections. Moreover, only immunohistological methods provide specific detection of microorganisms. These techniques use monoclonal or polyclonal antibodies directed against specific microbial antigens. Polyclonal antibodies are produced by different cells and, as a consequence, are immunochemically dissimilar. They react with various epitopes on the antigen against which they are raised. The animals most frequently used for the production of polyclonal antibodies are rabbits and goats (Lepidi et al. 2000, 2003b, 2004). Several other animals can be used to raise polyclonal antibodies. In contrast, monoclonal antibodies are produced

by clones of plasma cells. Antibodies from a given clone are immunohistochemically identical and react with a specific epitope on the antigen against which they are raised. Mice are currently used almost exclusively for the production of monoclonal antibodies. Once bound, the antibodies are detected by use of either fluorescent or chromogenic signal amplification. Immunofluorescence methods are usually performed on freshly frozen tissue, whereas immunoperoxidase methods are usually performed on formalin-fixed, paraffin-embedded tissues. These methods are useful for the detection of fastidious or noncultivatable microorganisms, or when the tissues have been fully fixed, for differentiating between morphologically similar microorganisms or cytopathic effects, and for the detection of highly infectious microorganisms involved in outbreaks of infection. Detection of fastidious microorganisms by use of ancillary methods is particularly important because they may go undetected in the microbiology laboratory. For example, *Coxiella burnetii* or *Trophyrema whipplei*, the causative agents of Q fever and Whipple's disease, respectively, are usually not cultured, but they can be readily detected in tissue samples from infected patients by use of immunoperoxidase methods (Lepidi et al. 2002b, 2003a, 2003b, 2004). The specificity imparted by immunohistological stains has been used to differentiate morphologically similar microorganisms such as *Rickettsia conorii* and *Rickettsia africae* (Lepidi et al. 2006). Similarly, these immunohistochemical methods have been used to differentiate morphologically cytopathic effects, such as those produced by *T. whipplei* and *Mycobacterium avium* or *Mycobacterium intracellulare* (Lepidi et al. 2003a). Immunohistology may also be more sensitive for detection of microorganisms that are difficult to locate in histologic sections (Toulaymat et al. 1999).

A potential pitfall of immunohistological methods is the failure to detect microorganism antigen because of prolonged storage of the tissue in fixatives such as formaldehyde. In these cases, additional steps for antigen retrieval must be performed, such as protease digestion, or heating in a microwave oven or in a 95–99°C water bath with sodium citrate buffer. However, specific antibodies for immunohistological staining are commercially available for a few bacteria. Polyclonal mouse or rabbit antibodies against microorganisms can be generated in laboratories if microorganisms are cultivated (Lepidi et al. 2000, 2003b, 2004, 2006).

Immunohistochemistry and immunofluorescence have been successfully employed in a number of palaeopathological studies, demonstrating that antigenic properties in ancient tissues can be preserved, and that preservation is related to different mummification processes and body storage conditions (Bruschi et al. 2006; Ciranni et al. 1999; Fornaciari and Marchetti 1986; Fornaciari et al. 1989). Immunohistochemistry can also be applied to old paraffin blocks in which antigenic epitopes are well preserved. This method has been employed to detect bacteria such as *T. whipplei* and *Rickettsia rickettsii* in one-century-old paraffin blocks from autopsy cases, or viruses and parasites in mummies (Bruschi et al. 2006; Dumler 1991; Dumler et al. 2003; Fornaciari and Marchetti 1986). However, although immunohistological methods seem an attractive option with which to detect and visualise microorganisms in ancient tissues, antigenic determinants in such tissues are often impaired or destroyed. This important technical limitation probably explains the very few studies concerning the detection of past pathogens in the literature.

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