Immunomicroscopy: A Diagnostic Tool for the Surgical Pathologist

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Immunomicroscopy: A Diagnostic Tool for the Surgical Pathologist

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To my wife Susan for laughing, and not laughing, in the right places and to Emma, Ben, Jeremy, and Matt for avoidance of major physical and emotional trauma during this labor.

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Foreword

Progress in the field of anatomic pathology has been shaped in large part by the tools—gross examination, light microscopy, histochemistry, electron microscopy, and so forth—available to researchers at the time of their investigations.

Immunochemistry, which has its origins in the techniques of immunofluorescence, has relatively recently emerged as an important and powerful diagnostic tool following the development of immunoenzymatic, particularly immunoperoxidase, techniques and the demonstration that their use extended to routinely fixed, paraffin-embedded tissues. Dr. Clive Taylor, the author of this volume of the Major Problems in Pathology series, has played a major role in the development of this new field.

New discoveries are being made each day in the applications of immunocytochemistry to the diagnosis of neoplasms and infectious diseases. The immunoperoxidase technique has already proved an invaluable aid to the classification of neoplasms in terms of the cell of origin, direction of differentiation, and biologic activity. It holds great promise for further use in the characterization of a variety of tumors through elucidating unique immunophenotypic signatures.

In view of the extensive research on immunochemistry in pathology during the past few years, it is essential that the results of these studies be critically reviewed and summarized in a single reference source. Dr. Taylor provides in IMMUNOMICROSCOPY an authoritative review of the subject of immunochemistry that is encyclopedic and also is enjoyable reading. The monograph amply covers both the theoretical and practical aspects of the subject. It will be invaluable to pathologists, oncologists, surgeons, microbiologists and basic scientists.

James L. Bennington, M.D.

Preface

"Begin at the beginning, go on until you get to the end, then stop."

Advice from the King to Alice in her Adventures in Wonderland.

LEWIS CARROLL (1832–1898)

This book sets out the basic theoretical and practical aspects of immunohistology and immunocytochemistry. The content and format is designed for use primarily by the practicing surgical pathologist and histotechnologist, rather than for the research scientist or the avant garde investigator. Dedicated researchers already have a sufficiency, even a surfeit, of reading material in the recent literature, which includes some 1000 immunoperoxidase references in 1984 alone, not to mention countless reports describing the various scientific pursuits of the adepts of the immunofluorescence school. The majority of these papers are highly specialized in content, dealing with only one of many minutely focused research interests or with only one or another of the principal immunohistologic techniques. Such works, although fundamental to the advancement of knowledge and technique in immunohistology are, by their specialized nature, of limited value to the practicing surgical pathologist wishing to adopt and adapt these methods to more routine but clinically immediate purposes. This book is intended to provide a selected literature resource and a working text for the practicing pathologist, a text sound in theory and explicit in practical details, with ample discussion of interpretation and usage of immunohistologic techniques in diagnostic practice.

Of the basic immunohistologic techniques, immunofluorescence is the older (45th birthday this year) and the better established. Immunoperoxidase methods, although conceived more than 20 years ago, suffered a long gestation period and really only emerged into the light of day in 1974, with the demonstration of applicability of immunoperoxidase methods to routinely processed paraffin sections. This book seeks to present the basic principles of both the immunofluorescence and immunoperoxidase methods, with a marked emphasis on the latter. The similarities between these two techniques are greater than the differences, yet the differences may lead to a preference for one method over the other, according to the demands of the study in hand. These same differences have also resulted in long and acrimonious debates, which have borne little fruit in proportion to the time and energy consumed.

The author of this book has had experience with both immunofluorescence and immunoperoxidase methods, has been fortunate to work directly with several of the pioneers in this field, and has rubbed shoulders with many others.

Chapter 1 deals with general principles, many of which apply both to immunoperoxidase and to immunofluorescence techniques, and include discussion of these methods in general terms. Subsequent chapters are divided into two groups; Chapters 2 and 3 describe technical aspects of fixation and the various immunoperoxidase techniques, and the remainder of the book is devoted to the practical applications of immunohistologic methods in diagnostic pathology. The rationale for selecting one method over another is given and discussed together with details of practical techniques and interpretation of the stained preparations. Certain key references are included within the text. However, most references are collected into tables covering particular topics; here the references are given by author and year with a brief commentary as to the relevance of the paper to the practicing surgical pathologist. This course has been adopted to achieve an uncluttered text from which the basic information pertaining to diagnosis may readily be extracted without wading through a morass of citations. The text presents a distillate of these tabulated references; access to the unadulterated original papers is given in a comprehensive bibliography at the end of the book. The appendices contain detailed staining protocols and information regarding commercial sources of the basic reagents and antibodies.

A HANDBOOK FOR EXPLORERS

Equipped with this book and a judicious selection of these wares, the pathologist may, without undue trepidation, venture forth into the still only partially explored world of immunohistochemistry. It will be something of an adventure. This is intended to be a guidebook; like all early guidebooks, it will be subject to repeated modification as the frontiers are defined and redefined. You, as co-adventurers are invited to share in this process.

CLIVE R. TAYLOR

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Contents

Chapter

1

PRINCIPLES OF IMMUNOMICROSCOPY

"The pathologist with the highest CQ (credibility quotient) often carries the day."

TAYLOR AND KLEDZIK, 1981

HISTORICAL BACKGROUND

The Microscope in the Beginning

The history of medicine is in large part the history of advances in basic sciences and the translation of these advances into diagnostic or therapeutic practice.

In the beginning there were only "practitioners of the art," variously termed physicians or barber surgeons, according to their background and proclivities. Pathologists existed only to the extent that a practitioner piqued by curiosity or morbid interest might, when all else had failed, seek the final solution at the autopsy table. Indeed, by means of the meticulously detailed autopsy (Fig. 1–1A), with painstaking attention to abnormalities of gross anatomy, the "art" was advanced ever so slowly over a period of several thousand years from ancient to modern times.

In this context, at least with reference to pathology, the modern era commenced in the middle of the nineteenth century. It is easy to forget that Hodgkin's disease, a condition now diagnosed on the basis of minutely described microscopic characteristics, was first recognized 150 years ago (by Thomas Hodgkin, 1832) on the basis of detailed gross dissection of seven cases, without resort to microscopic examination. Indeed, it was not until a decade later, in 1842, that one of the first organized courses in histology was given, by John Hughes Bennett at the University of Edinburgh. Microscopes and microscopists certainly existed prior to this time, as evidenced by the extensive botanic illustrations of Robert Hooke (*Micrographia*, London, 1665); the zoologic, principally entymologic, drawings of Jan Swammardam (*Bybel der Na tuur*, Utrecht, 1669); and the 247 microscopes that Atonj van Leeuwenhoek (1632–1723) is reputed to have possessed. Some have argued that the beginnings of histology date from the work of Marcello Malpighi (1628–1694), who delved into the microscopic world of embryology, portrayed the minute architecture of the spleen, and described red blood cells as "fat globules looking like a rosary of red coral," all this only a few years after Swammardam's first report of the existence of red corpuscles. However, these studies although startling in detail consisted only of accumulated sporadic observations and lacked the basis of an organized background of knowledge that Hughes Bennett was able to bring together for his histology course.

If Hughes Bennett deserves credit for his role in introducing histology into the curriculum and the minds of students of medicine, then Rudolf Virchow must be given credit for the first textbook of histopathology. Virchow's book, *Cellularpathologie*, was first published in 1856, with an English edition appearing in 1860 (Fig. 1–1B). It was based on a series of 20 lectures in which Virchow demonstrated, for the first time, that it was possible to establish the diagnosis and prognosis in many disease states by careful study, using the microscope, of cells and tissues. The concept that diseases resulted from imbalances of the body humors crumbled as physicians turned the pages of *Cellularpathologie* and were introduced to a new field of investigative and diagnostic medicine that was to form the foundation of histopathology.

The literature pertaining to microscopic anatomy and microscopic pathology grew rapidly as medical practitioners and researchers utilized the microscope to refine and redefine "old diseases" or to recognize and define hitherto undiscovered "new entities." Various staining techniques were developed

A

CELLULAR PATHOLOGY

AS BASED UPON

PHYSIOLOGICAL AND PATHOLOGICAL HISTOLOGY.

TWENTY LECTURES

DELIVERED IN THE

PATHOLOGICAL INSTITUTE OF BERLIN

DURING THE

MONTHS OF FEBRUARY, MARCH, AND APRIL, 1858.

BY

RUDOLF VIRCHOW,

PUBLIC PROFESSOR IN ORDINARY OF PATHOLOGICAL ANATOMY, GENERAL PATHOLOGY AND THERAPEUTICS, IN THE UNIVERSITY OF BERLIN; DIRECTOR OF THE PATHOLOGICAL INSTITUTE; AND PHYSICIAN TO THE CHARITÉ HOSPITAL, ETC. ETC.

TRANSLATED FROM THE SECOND EDITION OF THE ORIGINAL,

BY

FRANK CHANCE, B.A., M.B. CANTAB.

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WITH

NOTES AND NUMEROUS EMENDATIONS, PRINCIPALLY FROM MS. NOTES OF THE AUTHOR,

AND

ILLUSTRATED BY 144 ENGRAVINGS ON WOOD.

LONDON:

JOHN CHURCHILL, NEW BURLINGTON STREET.

MDCCCLX.

B

Figure 1–1. ***A,*** Boccaccio's vision of the "University of Ancient Rome" showing the Emperor Nero presiding at the autopsy of his mother Agrippina, whom he had put to death. (Original miniature in *Le Cas des Nobles et Femmes* [c1410]. Used with kind permission of Photographie Giraudon, Paris.) ***B,*** Title page for Cellular Pathology by Rudolf Virchow, English Language Edition, 1860. (Reproduced from a special edition issued by the Classics of Medicine Library, Birmingham, Alabama, 1978, by permission.)

to facilitate the study of tissue sections, staining cell nuclei, cytoplasm, or extracellular tissue components in a variety of contrasting colors. With each new report describing each new entity, the scope of histopathology was enlarged, with the result that within a few decades a major commitment of time and energy was required in order to become proficient in interpretation of the histologic appearances of normal and abnormal tissues. Thus the first pathologists emerged from the treacherous swamps of medieval medical practice onto the relatively firm ground that histopathology seemed to offer with respect to diagnosis of disease.

The practice of diagnostic histopathology, once established, changed little in principle during the succeeding 100 years. To be sure, numerous new morphologic criteria, some subtle to a degree, have been described, and many new entities have been established. Yet the principles by which the histopathologist works and learns his trade have not changed significantly.

When first sitting at the microscope, the fledgling pathologist is faced with the problem of the recognition and nomenclature of each individual cell. What are the characteristic features that distinguish lymphocytes, erythroblasts, plasma cells, thyroid "parafollicular" C cells, and others?

Traditionally the pathologist has only two ways of seeking answers to these questions, only two ways of learning the art of "tissue diagnosis." First, he can show the slide to a more experienced colleague; the appropriate recognition criteria are then passed by word of mouth from pathologist to pathologist, from generation to generation. The second approach, at first sight more scientific in basis, involves a search of the textbooks or medical literature for a written description

that corresponds to the cell in question, or even better, for a photomicrograph of a cell that upon comparison appears so similar to the cell visualized with the microscope as to justify applying to it the designation given in the textbook legend.

It is not too difficult to remind ourselves that criteria passed by word of mouth represent nothing more than an expression of the opinion of the experienced pathologist and that this opinion in turn paraphrases the opinion of his teachers. It is, however, rarely remembered that descriptions and illustrations in textbooks also are expressions of opinion and that these opinions, given additional weight and credibility by their appearance on the printed page, become tantamount to established facts. It is remarkable that histopathology, founded upon criteria derived from subjective opinions formed during the interpretation of cellular and subcellular patterns, has proven so successful and reliable over the years.

Nonetheless, experienced surgical pathologists recognize that many diagnoses are hedged with uncertainty as a direct consequence of the subjective nature of the judgments that pathologists must make.

Although knowledge of histopathology has advanced considerably, the basic principles of diagnosis based upon examination of a tissue section have not changed significantly since the time of Bennett and Virchow. However experienced the pathologist, the application and interpretation of morphologic criteria remain subjective. Diagnosis by examination of a tissue section is therefore often hedged with uncertainty, and opinions of experienced pathologists may differ critically with regard to the designation and import of any particular lesion. In such circumstances, when opinions differ, the diagnosis is usually resolved according to the consensus of opinions of several consulting pathologists, or in deference to the opinion of the most prestigious or the most forceful of those consulted. Thus the pathologist with the highest Credibility Quotient (CQ) (Fig. 1–2) often carries the day, and the diagnosis is considered final; there is no recourse to independent techniques for testing the rightness or wrongness of the prevailing opinion, upon which the ultimate diagnosis was based. Taylor & Kledzik, 1981.

The Need for Special Stains

Pathologists have long since recognized their fallibility, although they have not always publicized it (reviews, Taylor, 1978c, 1983a). They have therefore sought more certain means of validating morphologic judgments. A variety of "special stains" were developed to facilitate cell recognition and diagnosis; most of these stains were based upon chemical reactions of cell and tissue components (histochemistry). In certain circumstances these histochemical strains proved to be of critical value in specific cell identification. More often they served merely to highlight or emphasize cellular or histological features that supported a particular interpretation without providing truly specific confirmation.

Immunohistology

The facility for performing a wide variety of truly specific special stains was available, at least in potential form, with the advent of a practical immunofluorescence technique (Coons et al, 1941). The immunofluorescence-labelled antibody method exploits the specific binding between antibody and antigen, utilizing antibody labelled with a visible marker (usually fluorescein isothiocyanate) as a visible probe for the presence of the corresponding antigen within tissue sections or cell preparations. The immunofluorescence

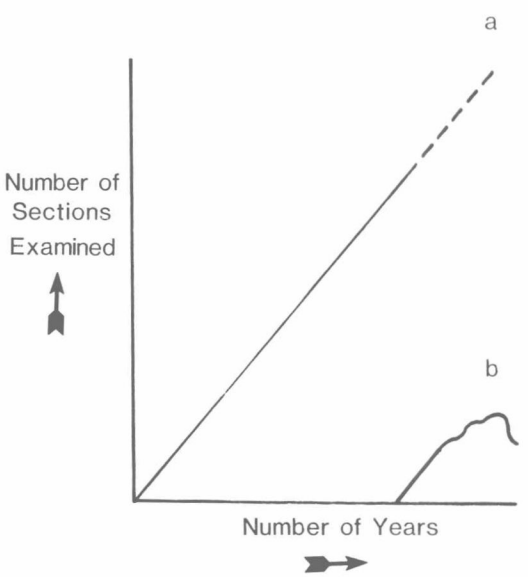

Figure 1–2. The workload accumulator graph (WAG) for calculating credibility quotient (CQ). WAG (a) shows a steady rise to infinity; the area under the line is a measure of the credibility quotient (CQ), which in this instance is awesome. WAG (b) represents a less impressive situation; the WAG has tailed off, the credibility quotient is correspondingly diminished, and the hedges of uncertainty loom tall in all directions. Type (b) WAGs stand to benefit most from immunohistologic methods; type (a) WAGs have the most to lose, particularly their aura of infallibility. It has been suggested that CQ and IQ show an inverse correlation.

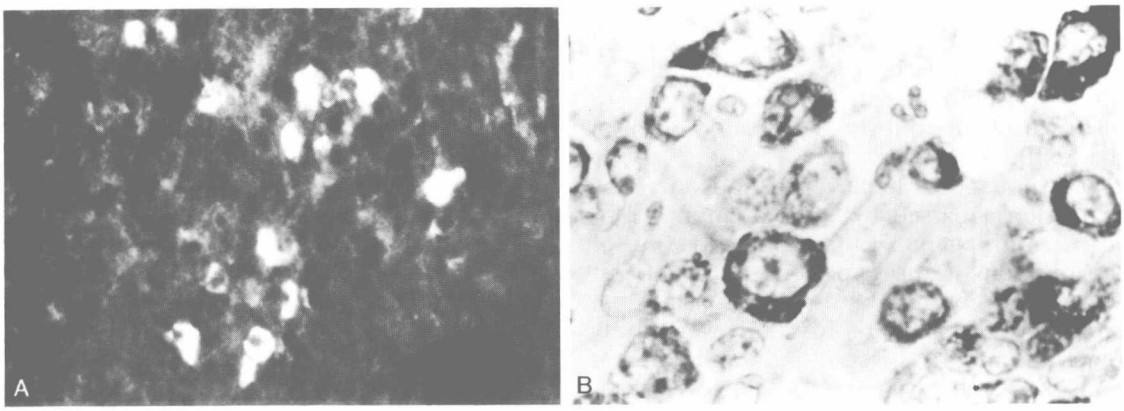

Figure 1–3. ***A,*** The immunofluorescent method on frozen sections; staining for IgG in plasma cells within a frozen section. Positive cells appear white. The morphologic features of positive and negative cells are not discernible. Frozen section, cold ethanol fixed (×320). ***B,*** The immunoperoxidase method on paraffin sections; portion of the same lymph node as in ***A,*** but fixed in formalin and embedded in paraffin. Staining for immunoglobulin is by the PAP method giving a positive black reaction in the cytoplasm. The chromogen is DAB (diaminobenzidine) the counterstain hematoxylin. The usual morphologic features upon which the pathologist relies for diagnosis are preserved. Note the diverse morphologic appearance of cells in the "plasma cell family," i.e., not many of the positive cells would be recognized as immunoglobulin-producing (plasma) cells by morphologic criteria alone (×900).

technique, popularized by the work of Coons (1941), Fagraeus (1948), and others, developed into a powerful research tool, generating an extensive literature. In time it provided the basis for the development of immunopathology as a flourishing, rapidly growing branch of investigative pathology. However, the immunofluorescence technique had little impact on diagnostic pathology as practiced by the surgical pathologist for reasons that relate principally to difficulties in applying the method to "routinely" processed paraffin-embedded materials.

Nonetheless, the success of immunofluorescence in pathology research created a pressing need for an alternative labelling system to avoid some of the disadvantages inherent in the immunofluorescent method, namely the need for specialized microscopy, application largely restricted to frozen sections and poor morphologic resolution (Taylor, 1983a) (Fig. 1–3A). Labels other than fluorescein isothiocyanate were sought. Of the many "labels" given trial, immunoenzyme techniques, linking antibody to an enzyme, in lieu of fluorescein isothiocyanate, proved most practical. Several enzymes were assessed in this system, including horseradish peroxidase, glucose oxidase, and alkaline phosphatase, together with a number of different substrate systems, for demonstration of the site of localization of enzyme-labelled antibody within tissue sections. Horseradish peroxidase in conjunction with diaminobenzidine and hydrogen peroxide as the substrate had advantages over other systems and became widely used.

The final critical step in making immunohistologic methods available to the surgical pathologist was taken in 1974, when it was shown that, using immunoperoxidase techniques, it was possible to demonstrate at least some antigens in routinely processed (formalin-paraffin) tissue (Fig. 1–3B). This discovery gave a new impetus to the use of immunohistologic methods. For the first time it was possible specifically to demonstrate a wide range of tissue components (antigens) in the types of tissues that the surgical pathologist has at his disposal when the need for special staining techniques becomes apparent in attempting to reach a diagnosis.

Only 10 years have passed since the feasibility of performing immunohistologic studies on routinely processed tissues was demonstrated with reference to the staining of immunoglobulin within plasma cells (Taylor, 1974; Taylor and Burns, 1974; Burns et al, 1974). In this decade, however, the range of antigens demonstrable in such tissues has increased enormously (Table 1–1); the literature contains reports of studies demonstrating more than 100 different antigens, many of which are relevant to the surgical pathologist as potential "special stains" with true specificity.

Table 1–1. Range of Antibodies That Have Been Successfully Employed for Demonstration of Antigens in Paraffin Sections

Hormones
ACTH
GH
LH
LTH
FSH
TSH
Parathormone
Calcitonin
Thyroxine
Thyroglobulin
hCG
beta-hCG
Testosterone
Estradiol
Progesterone
Insulin
Glucagon
Somatostatin
VIP
Gastrin
Secretin
Motilin
Neurotensin
Cholecystokinin
Renin
Vasopressin
Oxytocin
Neurophysin
Bombesin

Receptors
Transferrin receptor protein
Estrogen receptor protein

Other Tissue Components
Laminin
Fibrinectin
Collagens
Amyloid A protein

Other Cellular Components
Lysozyme (muramidase)
Lactoferrin
Transferrin

Other Cellular Components *Continued*
Ferritin
Hemoglobin A
Hemoglobin F
Myoglobin
Actin
Myosin
Keratin
$alpha_1$-Antitrypsin
$alpha_1$-Antichymotrypsin
alpha-Fetoprotein
Carcinoembryonic Ag
Mammary epithelial membrane Ag
Hepatorenal Ag
Pancreatic Ag
Melanoma Ag
Prostatic acid phosphatase
Prostate specific Ag
Glial fibrillary acidic protein
Tyrosine hydroxylase
Myelin basic protein
Enkephalin (-like) Ag
Substance P
Enolases
Adenosine deaminase
Terminal transferase
Carbonic anhydrase
Cathepsin D
Converting enzyme
Factor VIII-related Ag
Creatine kinase
Intestinal mucin Ags
HLA Ags
Blood group Ags
Surfactant apoprotein
S100 Ag
Neurofilament
Vimentin
Desmin
Mucin Ags
Celomic Ags
CALLA
CLA
Endocrine granule Ags
Ia-like Ags
B cell Ags
Monocyte Ags

Infectious Agents
Herpes I & II
Hepatitis B surface Ag
Mouse mammary tumor virus Ag
Rubella
Baboon endogenous virus
Measles Ag
Respiratory syncytial virus
Buffalo pox
Legionella
Kliebsiella
Group B streptococcus
Influenza
Poliovirus
Varicella-zoster
Cytomegalovirus (CMV)
Parainfluenza virus
Lymphocytic choriomeningitis virus
Moloney virus
Friend virus
Shope fibroma virus
SV 40
Human papilloma virus
Distemper virus
Rotavirus
Polyoma virus
Chlamydia
Mycoplasma
Trichomonas
Toxoplasma
Trichophyton
Amebae
Mycobacteria
Leishmania
Fasciola
HTLVI

Immunoglobulin Components
Kappa, lambda light chains
Gamma, alpha, mu, delta, epsilon heavy chains
J chain
Secretory component
C1, C2, C3, C4