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## Preface

The aim of *Hematologic Malignancies: Methods and Techniques* is to review those methods most useful for the diagnosis and subsequent management of hematologic malignancies. The scope of coverage is intentionally broad, ranging from routine procedures to highly sophisticated methods not currently offered by most clinical laboratories. The latter methods were selected especially to bring into focus recent advances in molecular biology that, since they provide us with strong tools for assessing the outcome of upcoming therapeutic modalities intent on disease eradication, are expected to impact the future diagnosis and management of these diseases. Thus, the common thread among all chapters is clinical relevance, whether sanctioned by past experience or by the expectation that seemingly esoteric research techniques of today will prove clinically valuable in the future. *Hematologic Malignancies: Methods and Techniques* is primarily a compilation of methods presented in sufficient detail—by authors with extensive expertise in their field—to serve not only as a reference for seasoned research and clinical laboratory personnel, but also as a guide for the less experienced. Moreover, the contributing authors also discuss the pathophysiologic bases and the diagnostic usefulness that underscore each method's clinical relevance. Thus, this volume should be also valuable to clinicians—especially hematologists, oncologists, and pathologists—often bewildered by an ever increasing flow of new scientific information, the practical application of which is often either not clearly disclosed or difficult to discern. Though the methods described in this volume are often used in combination for greater clinical impact, they are assembled here in 16 chapters grouped along five major themes: Cytogenetics; Polymerase Chain Reaction; Flow Cytometry; Cytochemistry and Immunohistochemistry; and Apoptosis and Cytokine Receptors.

Since the recognition by Tjio et al., in 1956 (*1*), that the human nuclear genome was contained in 46 rather than 48 chromosomes, the study of cytogenetics has evolved from the “dark ages” that preceded the development of the isolation and staining techniques necessary to identify individual chromosomes, to the current “molecular” era, in which detecting and locating specific genes, gene mutants, and gene rearrangements are possible through the use of DNA

probes. In Part 1, Drs. Sandberg, Baudis, and Hilgenfeld and their respective colleagues describe current methods for routine cytogenetics and fluorescence *in situ* hybridization (FISH), comparative genomic hybridization, and the recently introduced spectral karyotyping, respectively. They also review the clinical correlates of these techniques most useful for clinicians. Part 2 begins with Dr. Ward et al.'s review of those current methods in molecular biology and genetics that are most useful in the diagnosis and management of leukemia and lymphoma. This is followed by examples of applications of PCR for diagnosing T- and B-cell lymphomas, for detecting minimal residual leukemia, and for identification of the *NPM-ALK* fusion gene generated by the t(2;5) in non-Hodgkin's lymphoma, written by Drs. Sykes, Brisco, and Shurtleff and coauthors, respectively. Evolution from banding to PCR has expanded the precision and detection limits of cytogenetics analysis to approximately  $10^{-5}$ . The exquisite detection level and discriminant power of the newer techniques have propelled the Human Genome Project, a multinational public and private endeavor from which enormous scientific and medical benefits are expected, including a clearer understanding of gene function and regulation, and the detection of prenatal and preclinical disease, since it provides both the framework and a database for development of new strategies for disease prevention and control. Clinical benefits from these techniques are quickly materializing, as demonstrated by recent reports addressing cancer diagnosis and treatment. Using DNA microarray technology to determine gene expression profiling, a recent study demonstrated molecular heterogeneity in diffuse large B-cell lymphoma, as defined by histological and immunohistochemistry studies (2), a finding that might have an impact on clinical prognosis. Indeed, though 76% of 42 previously untreated patients expressing genes characteristic of germinal center B-cells were alive five years from diagnosis, only 16% of patients expressing genes normally associated with *in vitro* activation of blood B-cells survived five years. Heterogeneity uncovered by molecular fingerprinting is likely to explain sharp differences in the response rates and prognosis of patients with otherwise homogeneous cancers, and to guide the development of targeted therapeutic strategies. Probably the first example of a designed gene function regulation strategy for cancer management is the use of the oral *Bcr-Abl* tyrosine kinase inhibitor STI571 in chronic myelogenous leukemia (CML). The hallmark of CML is the Philadelphia chromosome, characterized by replacement of parts of *c-Abl* (at 9q34) with *Bcr* sequences (at 22q11). The resulting *Bcr-Abl* fusion gene encodes a protein product with enhanced tyrosine kinase activity that is pivotal in transforming transfected cells and confers on them a proliferative advantage. In recent phase I/II clinical trials (3), 96% of 54 interferon-resistant CML patients treated with STI571 in daily

doses exceeding 300 mg achieved complete hematologic remissions, including 33% cytogenetic responses, with minimal toxicity.

In 1972, Herzenberg et al. designed the first fluorescence-activated cell sorter (4). Since then, flow cytometry (FCM) has evolved from being primarily an expensive research tool requiring high maintenance instruments, to a highly versatile technique with increasingly widespread uses in many biological and medical fields. As retraced by Dr. Marti et al., in Part 3, technical factors that contributed to this evolution include: the rapidly evolving hybridoma technology that has generated a myriad of monoclonal antibodies (MAbs) that recognize a large array of surface and nuclear antigens on hematologic and nonhematologic cells; the increasing understanding of lymphocyte subset differentiation and of the complexity of interacting interleukins; progress in fluorochrome technology that allows the simultaneous use of distinctly conjugated MAbs with different specificities, thus enabling discriminant analysis of complex cellular components within fluids and tissues, and refinement and simplification of the hardware and software involved. More recently, the widespread use of FCM has been propelled by its suitability for assessing CD4 count in HIV, CD34 in bone marrow transplantation, and for a host of other prevalent clinical applications, including phenotyping, cell cycle and chromosome analysis, cell subset count and sorting, and functional studies. Given its sensitivity and specificity, and the biologic significance of its findings, FCM is viewed today as an indispensable tool for diagnosing hematologic malignancies and for monitoring their progress and response to therapy. Drs. Braylan and colleague detail the methods utilized in their reference laboratory for detecting surface and nuclear antigens, and for cell cycle analysis. Drs. Mosiman and Goolsby review the present status of what is sometimes referred to as “molecular cytometry,” a powerful technique that by combining FISH and FCM enables detection of DNA sequences of interest in cell population subsets defined by FCM. Finally, Dr. Vogt et al. address quantitative FCM issues that directly impact QA/QC and are finding increasing clinical usefulness as differing levels of antigen expression by normal and malignant cells are often associated with discriminant diagnostic and prognostic implications.

Cyto- or histochemistry, the technique of applying chemical stains to enhance microscopic differences among cells or tissues, originated with Francois Raspail in 1825 (5). Expansion of his initial observations and the systematic application of these “special stains” in the clinical setting were extremely slow. Furthermore, their lack of specificity and relatively low sensitivity reduce their usefulness so that they are now mostly used to confirm a particular interpretation, especially in surgical pathology, as described in Part 4 by Dr. Hanly, and led to the current immunohistochemistry era. This

latter approach exploits the specific binding between antibody and antigen, detected directly or indirectly by fluorescein- or enzyme-labeled primary or secondary antibodies, respectively. Demonstration of the power of this technique for distinguishing monoclonal from polyclonal disease in the clinical setting originated from our research laboratory. Using fluorescein-labeled antisera we identified a predominant monoclonal IgMκ cell population in histologically benign lymph node and involved lung tissue cryostat-frozen sections, in a patient with longstanding Sjögren's pseudolymphoma, 22 months before the lymphoma became clinically and histologically evident (6).

Though this initial approach was cumbersome and not easily adaptable to the clinical laboratory, developments over the subsequent two decades have led to the widespread adoption of immunohistochemistry, especially for the study of lymphomas. Contributing factors include: a broad range of MAbs directed against literally hundreds of antigens, the specificity of the binding reaction, and the advent of horseradish peroxidase-based techniques that allowed working with routinely processed tissues, thus enabling visualization of cellular constituents within well-preserved tissue sections. Yet, because antigens detected on malignant cells are also expressed by their normal, lineage-related cell counterparts immunohistochemistry assays are not disease-specific, with a few notable exceptions. Nevertheless, as Dr. Jaramillo et al. emphasizes, immunophenotyping helps detect evidence of malignancy, mainly through immunoglobulin light or heavy chain restriction, loss of pan-T or pan-B cell antigens, or aberrant cross-lineage antigen expression. Furthermore, distinct immunoarchitectural antigen expression patterns are characteristic of most lymphomas, a feature crucial in the differential diagnosis of these diseases. At present the concurrent assessment of immunohistochemistry, cytogenetics, and FCM has become the standard of practice in diagnosing and managing hematologic malignancies.

Part 5 presents overviews of apoptosis and of cytokine receptors analysis, written by Drs. Chiarugi and Zola, respectively. Apoptosis, or programmed cell death, is a normal physiologic process by which cells actively "commit suicide" and are removed by phagocytes, in sharp contrast to the process of necrosis that involves cell lysis followed by an inflammatory response. The discovery in 1985 of the *Bcl-2* was a milestone, for it provided the first example of an proto-oncogene that promotes clonal cell expansion by decreasing apoptosis rather than by promoting cell division (7). The best example of this is follicular B-cell non-Hodgkin's lymphoma, a slowly proliferative malignancy characterized by the presence of t(14:18). In this malignancy, the *Bcl-2* is translocated from chromosome 18 to 14, where it comes under the influence of immunoglobulin heavy chain gene-associated transcriptional elements that

lead to overproduction of Bcl-2 by malignant cells and, in turn, to decreased apoptosis. The process of apoptosis, regulated by gene-dependent counteracting influences that either promote or block cell death, has great potential relevance to cancer. Indeed, numerous types of cancers exhibit an overexpression of *Bcl-2*, several homologs of the *Bcl-2* family have been discovered in certain tumor-associated viruses, and it is now clear that gamma irradiation, and most if not all chemotherapeutic drugs, initiate apoptosis. Thus, novel anticancer strategies can be devised to modulate the intracytoplasmic concentrations of pro- and anti-apoptotic members of the *Bcl-2* family in favor of the former. Cytokines are a large and complex family of soluble protein mediators secreted by leukocytes, and they affect the growth, activation, or function of cells of the immune, hematopoietic, and other systems through cell surface receptors. Though our understanding of their role in malignancy is still fragmentary, their value in cancer management is underscored by their successful clinical applications. In a supporting role, colony-stimulating factors and, to a lesser extent, IL11 have become the standard of care for shortening the severity and duration of postchemotherapy leukopenia and thrombocytopenia, respectively. Additionally, certain cytokines exhibit anticancer activity, as is the case of IFN- $\alpha$  that induces the highest complete cytogenetic remission rates in CML when given concomitantly with cytosine arabinoside. Most patients who remain in continuous cytogenetic remission for at least one year appear to be free of residual disease, as judged by RT-PCR-negative *Bcr-Abl*. Finally, cell surface cytokine receptors can be used for targeted therapy, as demonstrated by DAB<sub>389</sub>IL-2, the first such agent approved by the FDA for clinical use. This agent is an IL-2 receptor-specific ligand fusion-protein produced by expression of a recombinant fusion gene in *Escherichia coli*. The fusion product consists of the first 389 residues of diphtheria toxin, containing the catalytic and transmembrane domains, fused to human IL-2. Upon binding to putative high affinity IL-2 receptors, preferentially expressed by cutaneous T-cell (CTCL) and other lymphomas, the catalytic domain is endocytosed and released into the cytosol, inducing protein synthesis inhibition and, ultimately, to cell death. In recent phase I clinical trials (8), 37% of 35 heavily pretreated patients with CTCL responded to DAB<sub>389</sub>IL-2 doses ranging from 3 mg/kg/d to a maximum tolerated dose of 27 mg/kg/d, including 14% complete remissions. Several other targeted fusion-protein toxins and immunotoxins (antibody-toxin conjugates) have also demonstrated significant anticancer activity in early clinical trials. However, challenging pharmacological issues and substantial toxicity limit the therapeutic index and efficacy of this group of agents.

These are exciting times in which accelerating advances in bioscience and biotechnology are enabling us to address fundamental questions about the

nature, origin, and growth regulation of cancer. They provide increasingly discriminant tools for the detection of neoplasia at the molecular level, and have increased the feasibility of developing more selective anticancer agents and of designing rational strategies for its control and eradication. Although most initial steps involving these strategies have met with limited success, they do represent a welcome departure from traditional semi-empirical drug development and testing by solidly grounding cancer therapy in our evolving knowledge of cancer genetics, biology, and growth regulation. It is hoped that the information contained in *Hematologic Malignancies: Methods and Techniques* will not only prove helpful to all research workers and health care providers managing patients with hematologic malignancies, but also serve to entice a new generation of young scientists to consider a career in cancer research.

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<http://www.springer.com/978-0-89603-543-0>

Hematologic Malignancies

Methods and Techniques

Faguet, G. (Ed.)

2001, XV, 360 p., Hardcover

ISBN: 978-0-89603-543-0

A product of Humana Press