Milestones in Investigative Pathology

2001 - 2009

Editor
Richard G. Lynch, MD
Introduction

*Milestones in Investigative Pathology* is a collection of articles written for the American Society for Investigative Pathology (ASIP) newsletters over the past decade. The brainchild of Dr. Richard G. (Dick) Lynch, these *Milestones* articles briefly summarize seminal research findings during (primarily) the 20th century that have had an extraordinary impact on our basic understanding of biological processes, on our approach to the diagnosis and treatment of diseases, and on global healthcare. The original *Milestones* series appeared in *The ASIP Bulletin* (July 2000 – July 2006) and *ASIP Pathways* (November 2006 – April 2009), after selection of subjects by Dick, who in fact wrote almost all of the articles himself. Each individual *Milestones* article is available online at http://www.asip.org/pubs/milestones.htm, as is this compilation.

Dick Lynch has been a leader in investigative pathology and has provided invaluable service to the ASIP for over two decades. A former Chair and currently Professor Emeritus of the Department of Pathology at the University of Iowa's College of Medicine, Dick was first elected to the ASIP Council in 1990 and served as President from 1995 to 1996. After serving as President, Dick represented ASIP and the discipline of pathology in the public affairs arena through the Federation of American Societies for Experimental Biology (FASEB) Public Affairs Committee for many years and contributed to and edited FASEB's *Breakthroughs in Bioscience* publications – a series designed to educate the general public about the benefits of fundamental biomedical research. Dick continues to participate in ASIP Council meetings today, serving as a trusted advisor to the elected leadership and as an active member of several committees. Dick received the ASIP Rous-Whipple Award in recognition for his scientific accomplishments in 1997. The photograph we selected for this compendium is, as he puts it, an “earlier vintage Dick Lynch” with a background montage of electron micrographs of his own research on myeloma cells.

Several common themes emerge from the Milestones articles, which span the gamut of diseases, including infectious, inherited, neoplastic, cardiovascular, and immunologic, among others:

- Experimental model systems have been essential to scientific discovery.
- Deciphering the pathogenesis of diseases advances our understanding of central biological processes.
- Many of the seminal discoveries that have impacted on 21st century science were not immediately accepted by the scientific community when they were first made.
- Trainees working with seasoned mentors have played a major role in the milestone discoveries.

It is our hope that the *Milestones* compendium will serve as an inspiration to beginning investigators in their quest to develop the scientific milestones of the 21st century.

Mark E. Sobel, MD, PhD
Executive Officer
September 30, 2009
<table>
<thead>
<tr>
<th>Topic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction by Mark E. Sobel, MD, PhD</td>
</tr>
<tr>
<td>The Philadelphia Chromosome</td>
</tr>
<tr>
<td>Tumor Stem Cells</td>
</tr>
<tr>
<td>Occult Carcinoma of the Prostate</td>
</tr>
<tr>
<td>Condylomata, HPV and Cervical Cancer</td>
</tr>
<tr>
<td>Cigarette Smoking and Lung Cancer</td>
</tr>
<tr>
<td>Surfactant and RDS in Premature Infants</td>
</tr>
<tr>
<td>Cell Theory and Neoplasia</td>
</tr>
<tr>
<td><em>Helicobacter Pylori</em> and Ulcers</td>
</tr>
<tr>
<td>Plasmacytomas and Basic Immunology</td>
</tr>
<tr>
<td>Coronary and Cerebral Thromboses</td>
</tr>
<tr>
<td>Lymphocyte Traffic</td>
</tr>
<tr>
<td>Immunoassays</td>
</tr>
<tr>
<td>Hypertension and the Kidney</td>
</tr>
<tr>
<td>The Neural Crest and Neurocristopathies</td>
</tr>
<tr>
<td>Pneumococcal Transformation: Genes are Made of DNA</td>
</tr>
<tr>
<td>Tissue Culture of Mammalian Cells</td>
</tr>
<tr>
<td>Pernicious Anemia</td>
</tr>
<tr>
<td>Apoptosis: Programmed Cell Death</td>
</tr>
</tbody>
</table>
The Philadelphia Chromosome

Richard G. Lynch, MD


MILESTONES

The milestone research reported by Nowell and Hungerford in Science in 1960 identified for the first time a consistent chromosome abnormality in a human neoplasm. Through meticulous examination of metaphase chromosome preparations they observed that one of the four smallest acrocentric chromosomes was markedly reduced in size, by what appeared to be the loss of approximately half of its long arm. The consistent association of this abnormality with chronic granulocytic leukemia suggested to Nowell and Hungerford that this chromosome change might confer on the leukemia cells their neoplastic character. A pathogenic role for the chromosome abnormality was further implied by its presence in leukemia cells at the onset of disease and prior to any treatment, and by its persistence in leukemia cells of patients whose disease had been present for many years. Investigators in Edinburgh who designated the abnormal chromosome the Philadelphia chromosome (Ph) confirmed the findings of Nowell and Hungerford. Peter Nowell was then, and still is, an investigative pathologist in the Department of Pathology at the University of Pennsylvania, and David Hungerford was a cytogeneticist at The Institute for Cancer Research in Philadelphia. A critical aspect of their research was the comparison of the chromosomes in the patients’ leukemia cells to the chromosomes in their normal cells. Although very labor-intensive, it was possible to visualize individual chromosomes in leukemia cells because these cells spontaneously divide when cultured in vitro, and in the presence of colchicine the mitoses are arrested in metaphase. Visualizing the chromosomes of the normal cells in the patients’ blood samples could have presented a barrier to this research because normal blood leukocytes do not spontaneously divide when placed in cell culture. However, in another fundamental discovery, Nowell had observed that phytohemagglutinin (PHA) – a plant mucoprotein used to separate leukocytes from erythrocytes in the blood samples – had the property of being a powerful lymphocyte mitogen. This discovery made it possible to prepare chromosomes from the normal, non-leukemia leukocytes present in the patients’ blood.

At the 1960 Fall Meeting of the National Academy of Sciences, Peter Nowell and David Hungerford reported that in each of seven patients with chronic granulocytic leukemia that they had studied, the leukemia cells contained an abnormal small chromosome that was not present in normal cells or in the cells of other types of leukemia. The abstract of their presentation appeared in Science and was followed by a more detailed publication describing their findings in ten patients with chronic granulocytic leukemia.

The discovery of the Philadelphia chromosome by Nowell and Hungerford was strong evidence that linked a genetic abnormality with human cancer. Their discovery was a landmark in cancer research and proved to be a seminal event in the field of cancer cytogenetics. Many thousands of publications and hundreds of research projects from numerous laboratories around the world can trace their ancestry to the original findings described by Nowell and Hungerford in their classic paper. A tremendous interest in the area of human cancer cytogenetics continues unabated to the present day, and the growth of knowledge in this area has been very impressive. The technology available to examine human chromosomes in 1960 only allowed for the detection of gross abnormalities in chromosome morphology and number. Compared to the penetrating, sophisticated molecular analyses used by investigators today, those tools were rather primitive. The introduction of the quinicrine fluorescence/Giemsa banding technique in the 1970’s was a major advance in cytogenetics and its application to the study of chronic granulocytic leukemia cells subsequently established that the Philadelphia chromosome was produced by a reciprocal translocation between chromosomes 22 and 9. In this abnormality a truncated portion of the protooncogene c-ABL from chromosome 9 relocates to the BCR gene locus on chromosome 22 and a large portion of the long arm of chromosome 22 relocates to chromosome 9. This reciprocal translocation
has two important consequences. The first is that it results in a significant reduction in the size of chromosome 22, the alteration that allowed Nowell and Hungerford to detect the cytogenetic abnormality in chronic granulocytic leukemia cells. The second consequence, which was elucidated later, was that the translocation resulted in the fusion of two genes, BCR andABL1, to form the hybrid oncogene BCR-ABL. It was subsequently shown that the fused BCR-ABL gene encoded a chimeric protein that has tyrosine kinase activity and is leukemogenic. The results of a recent clinical trial suggest that the pharmacological blockade of the BCR-ABL kinase may be of value in the treatment of chronic granulocytic leukemia.

The impressive growth of knowledge about the roles of genetic alterations in the pathogenesis of human leukemia and lymphoma that has taken place since the milestone discovery of Nowell and Hungerford has also revealed the enormous level of complexity involved in these processes. The degree of this complexity has significant implications for the treatment of these malignant neoplasms, a subject that has been discussed by Nowell.

References

Tumor Stem Cells

Richard G. Lynch, MD


MILESTONES


In ground-breaking research published in 1964, Barry Pierce, then a faculty member in the Department of Pathology at the University of Michigan, and L.J. Kleinsmith, a student fellow in his laboratory, demonstrated that single undifferentiated cells isolated from a murine teratocarcinoma, when transferred into normal mice, gave rise to malignant teratocarcinomas that contained differentiated tissues representative of all three major germ cell layers. When examined by light microscopy, the teratocarcinomas consisted of foci of undifferentiated malignant cells interspersed with disorganized arrays of adult somatic tissues representative of endoderm, mesoderm and ectoderm in various stages of differentiation. The differentiated tissues included neural, gastrointestinal, skin, muscle, bone, cartilage, marrow, notochord and yolk sac. By morphological criteria, the differentiated somatic tissues in the teratocarcinomas were considered to be nonmalignant. Pierce concluded that these murine teratocarcinomas were malignant tumors that consisted of a pool of replicating, multipotential tumor stem cells that gave rise to nonreplicating differentiated cells whose organization mimicked normal tissue development. Based on these findings and on histological features routinely observed in many human and experimental cancers, Pierce hypothesized that most cancers contained a pool of malignant stem cells, some of whose progeny differentiated into nonmalignant, post-mitotic tumor cells. In effect, Pierce was proposing that a malignant cell could become benign. This concept challenged the dogma "once a cancer cell, always a cancer cell," and Pierce found little enthusiasm for his concept amongst cancer researchers. At the time the studies were published it was generally believed by oncologists that teratocarcinomas were not representative of other cancers and, as interesting as these tumors might be, they were oddities and not relevant to cancer in general. There were several unique characteristics of murine teratocarcinomas that fostered this skepticism. Spontaneous testicular teratocarcinomas occurred only in the 129 strain of mice, these tumors developed in the gonad during fetal life, and it was possible to experimentally induce teratocarcinomas by injecting normal primordial stem cells from blastocysts of 129 strain mice into the testes of adult mice. Perhaps also operating in the background was the longstanding speculation by some scholars that teratocarcinomas actually reflected aberrant parthenogenetic embryogenesis, a concept that is likely related to their designation by some as embryos.

Pierce’s concept linking differentiation and cancer was supported by the startling finding of Leroy Stevens that the normal pleuripotent embryonic stem cells in a murine blastocyst, cells which if left in the blastocyst would develop into a mouse, developed into a teratoma or teratocarcinoma if they were injected into an adult testis. This finding appeared to complete a transformation circuit that linked tumorigenesis, embryogenesis and differentiation because, as already mentioned, Pierce had shown that cancer cells could give rise to normal differentiated adult cells. As a diagnostic pathologist, Pierce was aware of rare clinical instances in which highly malignant cancer cells in a patient appeared to spontaneously differentiate and the tumor regressed.

When such cases were reported in the literature, they were considered medical curiosities for which there was no explanation. An example is the report in The American Journal of Pathology in 1927 by Harvey Cushing and S.B. Wolbach that described a patient with a neuroblastoma in which the malignant neuroblasts spontaneously differentiated into mature ganglion cells to form a benign ganglioneuroma. Convinced of the merit of his concept, and having moved to the Department of Pathology at the University of Colorado, Pierce expanded the scope of his research to include investigations of other cancers besides the murine teratocarcinomas.

In a milestone paper published in Cancer Research in 1971, Pierce and Wallace used a
rat squamous cell carcinoma to test the hypothesis that the cancer contained a pool of proliferating stem cells and a pool of non-proliferating, post-mitotic differentiated cells. Microscopically this cancer consisted of foci of heavily keratinized flattened epithelial cells designated as "keratin pearls" that were reminiscent of the cells in the upper layer of normal skin. These foci were separated from each other by areas of undifferentiated cancer cells, many of which contained mitotic figures. When rats bearing this cancer were injected with tritiated thymidine and the tumors examined at various time intervals afterwards using light and electron microscopic autoradiography, it was observed that two hours after injection the thymidine label was present almost exclusively in the undifferentiated cells of the tumor. During the 96-hour period of observation there was a progressive increase in the number of labeled cells that were present in the highly differentiated areas of the tumor.

The investigators concluded that the cells in the keratin pearls were not synthesizing DNA and that the growth of the pearls depended on incorporation of undifferentiated cancer cells into the pearls with subsequent differentiation. The electron microscopic analyses expanded and confirmed these findings. The initial thymidine incorporation occurred in ultrastructurally undifferentiated cancer cells and later the label appeared in tumor cells that had desmosomes and other features of the cells of the normal stratum spinosum of skin. At even later times the label appeared in tumor cells that had features of granular layer cells of normal skin. In addition to these morphological findings, Pierce and Wallace microdissected undifferentiated areas and differentiated areas from the cancer and transplanted these into normal rats. Squamous cell carcinomas developed in about a third of the rats injected with undifferentiated cells, but in none of the rats injected with differentiated cells. In later studies, Pierce and colleagues investigated chondrosarcomas, and adenocarcinomas of the breast and colon and made comparable findings and conclusions.

In addition to the fundamental knowledge that these studies contributed to understanding the role of differentiation in cancer, they established the foundation of a novel strategy for treating cancers based on inducing the differentiation of malignant cells to a post-mitotic state. The investigations of Barry Pierce and Leroy Stevens in the murine teratocarcinoma model facilitated the discoveries by Brinster4 and by Illmensee and Mintz5 that the malignant stem cells of the 129 strain teratocarcinomas, when injected into normal blastocysts from other strains of mice, produced normal offspring that were genetic mosaics. Thus, the same tumor stem cells that produced teratocarcinomas when injected into adult testes were found to differentiate into the full range of normal adult tissues in the mosaic mice that were produced when injected into normal blastocysts. These findings eventually led to the development of 129 strain teratocarcinoma stem cells as tools for constructing transgenic6 and gene knockout mice. While considered by many in the beginning as non-relevant oddities, teratocarcinomas have yielded an abundance of fundamental knowledge about developmental biology and the pathobiology of cancer, and have contributed to the development of some of the most powerful genetic tools currently in use.

References
Occult Carcinoma of the Prostate

Richard G. Lynch, MD

In 1935, two autopsy-based studies by A.R Rich1 and R.A. Moore2 identified a surprisingly high incidence of latent prostatic cancer in elderly men. At the time these findings were published, they did not generate much excitement in the medical community, possibly because it was well known that pathologists always observed higher incidences of cancers than the clinical physicians. Many decades after their appearance as back-to-back articles in the Journal of Urology, these studies have taken on an enormous importance. It is unlikely that Arnold Rich, the famous Johns Hopkins pathologist, or Robert Moore, the eminent Cornell pathologist, ever imagined the extraordinary clinical and public health importance their findings would assume a half of a century later.

Rich became interested in latent prostatic cancer from his experience on the Hopkins autopsy service where he came to believe that clinically unsuspected prostate cancer was not uncommon, although in searching the literature, he was unable to find any publications that addressed this issue. Moore had spent time in Vienna where he had been encouraged to investigate latent prostatic cancer by the famous Austrian pathologist Erdheim. Rich's study consisted of examining a single routine histological section of the prostate gland from 292 consecutive autopsies performed on men 50 years of age or older. He found a 9% incidence of latent cancer, but assumed this was a minimum estimate because only a single random slide of each gland was available for study. Moore examined multiple step sections of 229 prostate glands removed at autopsy from patients over age 50 and found microscopic carcinoma in 20.5% of them. Both studies found that the incidence of clinically unsuspected prostate cancer increased with age and approximately doubled with each additional decade of life.

The articles by Rich1 and Moore2 are milestones because they were the first to identify a fundamental characteristic of prostate cancer that is highly relevant to the field of prostate cancer today. The astonishingly high incidence of latent prostate cancer has implications for research aimed at understanding the basic biology of human prostate cancer as well as for clinical investigations that address therapeutic decisions in response to the finding of an elevated serum PSA. Later studies by other investigators indicate that latent prostate cancer may have an incidence as high as 70 to 80% in men in their 80s and 90s. These extraordinary rates of latent cancer contrast with the 6 to 8% lifetime risk that individual men have of developing clinically diagnosed prostate cancer. This striking discrepancy indicates that about 90% of latent prostatic cancers remain clinically silent for decades. This conclusion is also supported by the findings of Bauer et al3 that when latent cancer was discovered in a suprapubic prostatectomy specimen and the patients were not treated for the cancer, their survival times were not different from the normal life span for age-matched males if the latent cancers were well differentiated. In another study by Greene et al4, when latent cancers were found in TUR specimens, the 5- and 10-year survival times for patients not treated for the cancer were 95% and 85%, respectively, of normal life expectancy for their age group. While the overall prevalence of latent prostate cancer at autopsy does not differ between blacks and whites in the United States, there is at least a two-fold higher incidence of progression to overt clinical cancer in black men.

The findings of Rich1 and Moore2 have direct implications for the dilemma that can be created for the patient and for the clinician when a microscopic focus of prostatic cancer is seen in a biopsy performed to evaluate an elevated serum PSA level. Is this one of the nine latent cancers that will remain silent for many decades, or is this the one in ten that will become clinically significant? Since we can not be sure, should we treat all of them as if they are the one in ten? These decisions are being influenced today by information that continues to come from the lineage of investigations that
trace their origins to the findings of Rich\(^1\) and Moore\(^2\). These studies have shown that prostate cancer, even when locally invasive of the perineural spaces in the prostate gland, can exist for decades as a latent process that is clinically inconsequential. The studies have also shown that the degree of differentiation of the latent cancer is a reliable predictor of its subsequent clinical behavior. Latent cancer of the prostate has an enormous public health and economic significance. About a decade ago when PSA was being recommended as an annual screening test for all males in the United States over the age of 50, the Department of Public Health in the State of New York estimated that the annual costs of the diagnostic and therapeutic procedures involved in the follow-up of elevated PSA levels were in the range of 26 billion dollars!

The studies by Rich\(^1\) and Moore\(^2\) were simple in design, completely descriptive, but powerful in their insight and impact. Descriptive studies often get criticized for their inadequacy, but they often are the starting point for a line of inquiry. Insights into the biology and mechanisms of disease continue to come from descriptive studies done in the clinic and in the autopsy suite. As Yogi Berra once said: “You can observe a lot by watching.”

References

Condylomata, HPV and Cervical Cancer

Richard G. Lynch, MD


MILESTONES


These two cited papers are landmark publications in the history of investigative pathology for several reasons, the most important of which is the profound and lasting impact they have had on women’s health. Today, we work in an era of biomedical research where there is a growing emphasis on "translational research." This term usually means advancing clinical practice through the application of knowledge generated in the basic research laboratory. The studies of Meisels and colleagues1,2 certainly had an immediate clinical impact, but their work is also an excellent example of translational research vectored in the reverse direction: observations made in the clinic stimulated basic research in the molecular genetics and biology of human papilloma viruses that advanced the field and continues to the present day. The investigations of Meisels employed an approach and a set of technologies, which by today’s standards would be considered rather simple. Their findings yielded fundamental insight into an important human cancer.

Compared to the numerous and often sophisticated tests that have been devised to detect the presence of a cancer, the simple technique for examining exfoliated cells in vaginal secretions described by the anatomist George Papanicolaou1 has no peer when measured in terms of a public health impact. Before the Pap smear came into widespread use in the United States, cervical cancer was the leading cause of death from cancer in women, but it now ranks eighth. This dramatic fall in mortality rate reflects the early and effective treatment of asymptomatic patients whose vaginal cytology showed the presence of cancer cells, or cells indicative of a pre-malignant lesion. Although the mortality rate from cervical cancer is now relatively low, approximately one million cases of precancerous conditions of the cervix are detected annually by Pap smears in the United States. In countries such as Viet Nam, where nationwide cytology screening does not occur, cervical cancer is the leading cause of death from cancer in women.

The investigations by Meisels and colleagues1,2 were milestone contributions because they established that papilloma virus infection of the cells that line the uterine cervix was very common and that it was a high risk factor for the subsequent development of cervical cancer. At the time these studies were published, it was already known that a papilloma virus was the etiological agent of Condyloma acuminatum, a sexually transmitted disease that, because of the appearance of the lesions, is commonly referred to as venereal warts. These are benign squamous cell tumors that occur on the external genitals and the perineal skin in females and males. Instances of malignant transformation of these lesions had been reported in the literature, but were rare. Epidemiologic studies had established that the risk factors for developing venereal warts were the same as the risk factors for developing cervical cancer. These included sexual promiscuity and the initiation of sexual activity at an early age. A characteristic microscopic feature of venereal warts is the presence of a striking paranuclear halo in some of the squamous epithelial cells. Cytologists had occasionally observed cells with paranuclear halos in vaginal smears from women who did not have a history of venereal warts, but these cells received little comment and their significance was unknown. The first published description of halo cells was in 1949 by J. Ernest Ayre, a pathologist at the Royal Victoria Hospital in Montreal, who considered them to be "precancerous cells". The conspicuous halo present in these cells prompted Koss and Durfee in a 1956 paper3 to designate them as koilocytes (koilos, the Greek word for hollow or cavity). Ayre4 was the first to mention in the literature that halo cells might be a manifestation of a viral infection. Meisels’ investigations5 established that the koilocytes present in vaginal secretions had cytological features in common with the halo cells present in venereal warts. In a very systematic study of otherwise normal women whose vaginal smears contained halo cells, Meisels and
colleagues detected focal alterations of the cervical surface which were rather inconspicuous and easily missed on routine clinical examination. Biopsies of these lesions revealed the presence of halo cells and other histological features indicating that they were flat or inverted condylomata. In contrast to the conspicuous papillary excrescences of condyloma acuminatum of the external genitals and perineal skin, Meisels discovered that condyloma of the cervix were usually tiny, flat non-specific lesions that could easily be confused with other types of epithelial lesions. Once Meisels established the cytological criteria for condyloma, it soon became clear that most vaginal smears previously diagnosed as cervical dysplasia were actually cases of cervical condyloma. In discussing their findings Meisels and colleagues proposed that cervical condyloma might be an early step in the natural history of cervical neoplasia. The age of peak incidence of cervical condylomas in their study was 19 years, which is a significantly younger age than the peak incidence for carcinoma of the cervix. Since cervical condylomata eventually disappear, they proposed that the virus becomes latent, but that “later in life if host factors become favorable (lower immunity against the virus, repeated local trauma, infections and infestations of the cervix) the virus, probably now integrated in the host genome, activates the mechanisms of carcinogenesis.” The essence of their proposal remains valid today.

During the decades that followed publication of these milestone articles, the field of human papilloma virus (HPV) research has grown tremendously. More than 70 different types of HPV have been identified from clinical specimens and it is clear that a large number of subtypes and variants exist for each type of HPV. There is now an abundance of evidence that links HPV to cervical cancer and its precursor conditions. HPV DNA is detected in 85% of cervical cancers and 90% of cervical condylomata. Distinct HPV types (“high risk” types) are associated with cervical cancer. It is known that in condylomata, HPV exists in an episomal (non-integrated) form but in cervical cancer the HPV is integrated into the host genome. The site in the human DNA where HPV integration occurs is random, but the site where the viral DNA is interrupted during integration is selective and results in the over-expression of two viral proteins that block the action of two host-cell proteins that are key regulators of the cell cycle. Currently, clinical trials are testing the efficacy of vaccines containing peptides from high-risk types of HPV as an immunotherapeutic strategy to prevent HPV-associated genital cancers.

Collectively, these are impressive advances in a field that is rapidly approaching a detailed molecular understanding of an important human cancer. The studies of Meisels and colleagues provided a tremendous impetus to this field. The simple methodology developed by Papinicoalaou set the stage. It is ironic that vaginal cytology, unquestionably the greatest success story in the field of human cancer, was initially met with such deep skepticism and strong resistance from within the discipline of pathology. Fortunately, the early practitioners of cytology persisted.

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Cigarette Smoking and Lung Cancer

Richard G. Lynch, MD

In these milestone articles published in the New England Journal of Medicine (NEJM), Oscar Auerbach and colleagues presented incontrovertible evidence linking cigarette smoking to the development of lung cancer in humans. The data presented in these publications resulted from a systematic, comprehensive and extraordinarily labor-intensive histopathological analysis of many hundreds of lungs obtained at autopsy. Dr. Auerbach was a pathologist at the Veterans Administration Hospital in East Orange, New Jersey and Professor of Pathology at New York Medical College. In the decades prior to Auerbach’s publications, epidemiological studies implicated cigarette smoking as an etiological agent in human lung cancer, a conclusion that was strongly supported by experiments conducted in laboratory animals. Nonetheless, the potential health hazard of cigarette smoking was a very controversial subject during the post-World War II years. Various interest groups challenged both the relevance to humans of experiments conducted in laboratory animals and the conclusiveness of population studies that showed statistical links between lung cancer and patterns of cigarette usage.

The Auerbach experimental protocol involved histological examination of samples taken from 208 specific sites of each tracheobronchial tree. All slides were coded and groups of slides from smokers and non-smokers were mixed and randomized so that the pathologist who examined the slides was totally ignorant of the clinical and smoking history of the patient. Individual lungs were scored for qualitative and quantitative mucosal alterations that included the loss of cilia, the presence of epithelial hyperplasia, and the occurrence of cytologic alterations that today would be categorized as degrees of atypia and dysplasia. It was well known from experimental studies that application of a carcinogen to a tissue caused cellular hyperplasia, metaplasia and nuclear and cytoplasmic atypia during the period before a tumor appeared. In designing their extensive sampling protocol Auerbach and colleagues assumed that if inhaled cigarette smoke contained a carcinogenic substance it would likely be widely distributed over the inner surfaces of the tracheobronchial tree and that cellular injury would also be widely distributed. They reasoned that if inhaled cigarette smoke was a major cause of lung cancer it would be expected that in smokers who died of lung cancer the remaining bronchial epithelium would manifest evidence of mucosal injury including hyperplasia, metaplasia and preneoplastic cytologic changes. They reasoned further that similar changes would occur in the bronchial epithelium of patients who died of other causes, but who had been heavy smokers. The data published in the NEJM in 1957 was a status report of Auerbach's large ongoing study and the 1961 NEJM article extended and refined the original observations. Auerbach had collected over 1500 tracheobronchial trees at autopsy and the results presented in each of the milestone articles reflected the microscopic examination of over 20,000 randomized and coded slides. The data from this enormous undertaking clearly showed that the frequency and severity of cellular alterations in the lungs were directly related to the extent and length of cigarette smoking by the patient. The lungs from non-smoking patients contained far fewer cellular alterations and these were always of a much lower grade than the lesions found in the lungs of smokers. There was excellent agreement between pathologists in scoring the slides for mucosal cell alterations.

During the 1950's the steady rise in number of deaths from lung cancer in the United States and in European countries – a pattern that had been noted as early as 1900 – and the increasing number of epidemiological studies implicating cigarette smoking as an etiologic factor in lung cancer led to a growing consensus by private and public health agencies in North America and Europe that cigarette smoking was a major cause of lung cancer.

Public declarations and policy statements by
such organizations as the American Cancer Society and the British Medical Research Council triggered counterarguments from individuals, groups and the tobacco industry who were critical of retrospective and population-based studies and skeptical about drawing conclusions from them. Auerbach’s studies moved the cigarette smoking - lung cancer controversy beyond an argument that was based on a statistical association. The extensive histopathologic analysis of individual lungs from many hundreds of patients, coupled with a thoroughly documented clinical and smoking history for each patient, allowed Auerbach to conclude in the 1961 NEJM paper: “In our opinion the histologic evidence from this study greatly strengthens the already overwhelming body of epidemiologic evidence that cigarette smoking is a major factor in the causation of bronchogenic carcinoma.”

The tremendous public health impact of the studies by Auerbach and colleagues is evident in the very prominent position they were given in the Report of the Advisory Committee to the Surgeon General of the Public Health Service and the very influential role they played in the subsequent actions taken by the Surgeon General regarding labeling and advertising of tobacco products. Auerbach’s work is a powerful example of the societal benefits that have come from investigations conducted with tissues obtained at autopsy, a resource that, unfortunately, is progressively disappearing.

References
Surfactant and RDS in Premature Infants

Richard G. Lynch, MD

MILESTONES


Kikkawa Y, Motoyama EK, Cook CD: The ultrastructure of the lungs of lambs. The American Journal of Pathology 1965, 47:877-903

The history of investigations that solved the mechanistic enigma of Respiratory Distress Syndrome (RDS) in premature infants is a splendid example of how multidisciplinary research drives medical progress. Until about 25 years ago RDS was a major health problem that affected approximately 25,000 newborns in the United States each year, with 10,000 of those infants dying early in the post-partum period. Today, RDS is uncommonly the cause of neonatal mortality in developed countries. This striking change is the result of research efforts that identified the pathologic mechanism underlying RDS and provided the scientific rationale for designing therapies that effectively prevent and manage this disease.

RDS in premature infants was commonly referred to as Hyaline Membrane Disease because at autopsy the lungs contained microscopic protein aggregates that formed characteristic membranes in the distal airways. Numerous theories were proposed to account for the origin and significance of the membranes, and for decades a prevailing view was that they consisted of debris present in amniotic fluid that had been aspirated by the fetus prior to birth. However, pathologists had long recognized that the most common autopsy finding in RDS lungs was severe atelectasis, the condition in which lungs are collapsed and airless, and pediatricians had observed that the degree of atelectasis progressively worsened as the clinical condition of the infant deteriorated. The obvious critical question was “Why did the lungs of premature infants become airless?”

Since early in the 20th century, physiologists and anatomists had speculated about the effects of surface tension in the air spaces of the lung. The pulmonary alveolus, the terminal anatomic structure of the lung, is a roughly spherical structure whose surface is a curved gas-liquid interface with physical properties that approximately fit the Law of Laplace: \( P = 2T/R \), where \( P \) is the pressure difference across the surface, \( T \) is the surface tension, and \( R \) is the radius of curvature. The Laplace relationship predicts an inherent instability of the pulmonary alveolus because if surface tension remains constant then the decrease in alveolar size upon expiration would increase intra-alveolar pressure and this would promote further decrease in alveolar size. To account for the observed stability of normal pulmonary alveoli, a surface active material (surfactant) capable of reducing alveolar surface tension had been proposed by some early investigators, but never demonstrated. In 1955, the English physicist R.E. Pattle published the results of experiments he had conducted on the stability of bubbles produced in various fluids including serum and pulmonary edema fluid. He observed a remarkable stability of the bubbles that were contained in the foam of pulmonary edema fluid compared to bubbles produced in serum and other fluids. He concluded that the bubbles of pulmonary edema fluid contained “a protein layer that can abolish the tension of the alveolar surface”. Further insight into the pathogenesis of RDS was provided in a presentation made to the New York Pathological Society in 1956 by Peter Gruenwald, a pathologist who worked at a maternity hospital in New Jersey. Based on autopsy findings, Gruenwald proposed that three factors contributed to the pattern of atelectasis of premature infants: a) a low ratio of capacity of alveoli versus bronchi in premature infants favors the loss of air from the alveoli when only part of the total volume of air leaves the lungs,” b) "in conditions of collapse following air breathing the respiratory surfaces have an increased adheriveness tending to perpetuate atelectasis caused by the first factor," and c) "surface tension favors the development of large bubbles in the bronchioles, rather than smaller ones in the alveoli." In 1956, J.A. Clements presented evidence for the presence of a surface tension-reducing material in lung extracts that was not present in serum or other tissues. This was soon followed by the breakthrough discovery of Avery and Mead that surfactant could not be detected in the lungs of infants who died with RDS, while it was readily detected in the lungs of infants who died of non-pulmonary disease.
provided their birth weight was greater than 1,000 grams. They proposed that prematurity and lack of surfactant were responsible for RDS.

The findings of Avery and Mead created widespread interest and stimulated investigations aimed at determining the chemical composition of surfactant, the site of its production, and the factors that regulated its expression. Numerous studies began to address these issues. An important set of observations was reported by Kikkawa and colleagues who studied respiratory distress in newborn lambs. In their model system, lambs delivered prematurely developed respiratory distress with loss of pulmonary surfactant, while full term lambs did not. Sequential electron microscopic analysis of the developing fetal lung revealed the appearance of osmiophilic inclusion bodies in certain alveolar lining cells (Type II pneumocytes) at about 121 days gestation. The number of inclusion bodies increased with further fetal maturation. Normal surfactant activity was first detected in lung extracts a few days after the initial appearance of the inclusion bodies. At the time this research was performed, the chemical structure of pulmonary surfactant had not been established, but it was known that the major component of surfactant was the saturated lipid dipalmitoyl phosphatidylcholine, an osmiophilic compound. The experiments of Kikkawa and colleagues confirmed the excretory properties of the Type II pneumocytes and the phospholipid nature of the osmiophilic inclusion bodies. These investigators concluded that the inclusion bodies were the source of surfactant because respiratory distress and loss of surfactant activity were associated with a decrease in the number and density of inclusions. In addition, the electron micrographs showed that the alveoli of normal lambs were lined by a dense osmiophilic layer, which implied that the secreted surfactant was distributed along the curved gas-liquid interface.

The research that solved the pathogenesis of RDS in premature infants resulted in the development of treatments aimed at preventing alveolar collapse. Surfactant replacement therapy and the use of ventilators to maintain a positive endrespiratory pressure were shown to be highly effective. The research also opened up an entirely new field of investigation that is ongoing. Much is now known about the biosynthesis, assembly and turnover of surfactant. The structures of four proteins present in pulmonary surfactant have been determined. Insights into the functions of these proteins have come from studies of mice with engineered mutations and of humans with inherited mutations. A growing number of publications have presented evidence that some of these proteins function in host innate immune responses, findings that implicate pulmonary surfactant in physiological functions that go beyond lowering alveolar surface tension.

References
The critical reader of the scientific literature knows it is the rare big idea that derives from the work of a single scientist. And yet many pathologists growing up in the second half of the 20th century, I among them, were willing to forego their skepticism and accept Virchow as largely, if not exclusively, responsible for the theory that all cells derive from pre-existing cells and do so by cell division.

A more realistic story begins with the promulgation of Theodor Schwann’s cell-theory. Although the facts supporting the generalization that all biological structures are comprised of cells did not originate with him alone, it was Schwann who convinced most of his peers that nuclei-containing cells were the universal, essential units of life. Schwann’s cell-theory also included his claim that the increase in number of most animal cells depended on the formation of new nuclei by a process akin to crystallization around nucleoli in an extracellular stuff he termed the Cytoblastem. Together with the rest of Schwann’s argument, his proposal for cell formation was widely adopted in spite of the lack of convincing, supportive microscopic evidence and reliable reports of cell division in protists and filamentous algae. In fairness to Schwann, the difficulties he faced as a microscopist in the late 1830s should not be underestimated. The compound microscope had come of age with the partial correction of chromatic aberration in achromatic objectives, but fixatives were limited, and neither embedments, microtomes nor differential stains were available. In any case, Virchow, like most others at the time, adopted Schwann’s story of animal cell creation wholeheartedly.

It was left to Robert Remak, following the lead of the botanists, to provide the initial evidence that binary division was responsible for animal cell replication rather than cell generation in a dubious extracellular slime. His initial observations were made on embryonic erythrocytes of the chick embryo. These studies were followed by investigations of developing muscle in the frog embryo, and in both he found cell forms that were consistent with binary cell division. Nowhere did he observe the extracellular, free nuclei demanded by Schwann’s cell-theory. Remak’s extensive embryological studies of chicken and dog published between 1851 and 1855, added to the evidence that cell division was the principal if not the sole means of new cell formation from the beginning of development in the fertilized ovum.

Remak, like Schwann and Virchow, trained in Johannes Müller’s laboratory at the University in Berlin. After earning his medical degree in 1838, he worked as an assistant to Müller and later Schönlein at the Charité Hospital, but, failing to be appointed to the position of prosector at the Charité, he found it necessary to support his family as a clinical neurologist, carrying out his embryological research and giving courses in microscopy in his apartment. An unconscionable delay in receiving an academic appointment in Berlin was the consequence of his unwillingness to forsake his Jewish religion, even as a formality. When his appointment at the University was finally approved, it carried with it neither salary nor laboratory.

The trajectory of Rudolph Virchow’s early career was in stark contrast to that of Remak. Virchow received his medical degree in 1843, and three years later was appointed prosector at the Charité, the position Remak had sought. Although six years Remak’s junior, the two were appointed to the faculty in Berlin in the same year, 1847. Suspended in 1849 from his University appointment for his political activity, Virchow was appointed professor at Würzburg. He returned to Berlin seven years later, again the victor in a competition with Remak, this time for the coveted appointment to a new professorship of pathology. Remak was not a strong contender for several reasons, not least among them his religion, but Virchow’ took no chances in pursuing the appointment, writing his father-in-law with advice for neutralizing Remak’s primary if not sole supporter,
Alexander von Humboldt, "As for Humboldt, he may perhaps be persuaded to change his mind. I do not know him personally, but Uncle Emil could certainly speak to him. Do you know any member of the Mendelssohn family? Since he (Humboldt) supports the Jews, one must use Jews to get the better of him." This proposal speaks more to Virchow's "Realpolitik" and some ambivalence towards the Jews rather than any overt antisemitism. Years later as rector of the University, Virchow would strongly oppose the antisemitism of the student organization and chide the faculty, "(Our age) still stands at a loss before the riddle of anti-Semitism... .Until now no one has asked for a professorship of anti-Semitism, but it is said that there are already anti-Semitic professors". He obviously did not consider himself one.

Remak summarized his early work on cell generation in an 1852 paper in which he reviewed the literature and summarized his own contributions, "Since the publication of the cell-theory, it has seemed to me that the extracellular creation of animal cells is as unlikely as the generation aequivoca (spontaneous generation). These doubts have led to my observations on the multiplication of blood cells by division in bird and mammalian embryos ......and the division of muscle bundles in frog larvae; then finally in the spring of 1851, I succeeded in finding that all embryonic cells multiply by division." He concluded, "These results are just as closely related to pathology as they are to physiology......I venture to suggest an apologetic for the limited number of examples, Remak still considered his findings together with the evidence in the literature sufficient to draw conclusions: "In general I find nuclei everywhere within cells and appearances which I consider to indicate an increase of the cells through division according to the mode I have described for normal tissues... I therefore now believe that the thesis can be formulated quite precisely that tumors are not new tissue entities but represent the transformation of normal tissues with growth by continued division in which either the structure and composition of the normal tissues persists (homology) or the structure and composition are modified through degenerative changes (heterology). Virchow14, reviewing the years' progress, reported on Remak's paper but was non-committal, acknowledging but neither rejecting nor accepting Remak's conclusion on cell formation in tumors.

In the same year in the first volume of the Handbuch of Speziellen Pathologie and Therapie25, Virchow offered his agreement with Remak that the formation of cells from free extracellular nuclei did not occur, but he was still unready to accept cell division as the exclusive mechanism of cell increase and listed four possible forms of cell replication: 1) the division of pre-existing cells, 2) cell budding 3) a modified form of Schleidin's proposed cell formation from intracellular particles, and 4) organization of an exudate or blood. Less than a year later, Virchow, in a bombastic editorial13 adopted the aphorism, omnis cellula a cellula, applying it to pathological changes including tumors. In the editorial, Virchow did not explicitly embrace cell division as the exclusive means of cell generation and provided no consideration of the mechanism; nor did he credit the work of Remak or anyone else for his modified position. Remak24 took grave offense at what he perceived as Virchow's failure to acknowledge his contributions, writing in a letter to Virchow, "In the first issue of the 8th volume of your archives the phrase: omnis cellula a cellula appears as your own without any mention of my name. That you make yourself ridiculous thereby in the eyes of the knowledgeable, since you have no evident embryological expertise, neither I nor anyone else can undo. If however you wish to avoid a public discussion of this matter, I would ask you to immediately acknowledge my contribution when and where you choose. It goes without saying that I reserve the right to judge if your clarification is sufficient in form and content."

Two years later in his published lectures, Cellularpathologie25, Virchow notes Remak's studies on erythroblasts and comments on his embryologic observations, "... if what has been most rigidly maintained by Remak is correct, namely that the cleavage of the yolk also is due to a visible division of cells, ...we are not dealing with a free organizing impulse working within the yolk but with progressive divisions of an originally single cell." Even then Virchow was not ready to accept Remak's concept of neoplasms arising from the various specific tissues of the body by progressive cell division, turning instead to the connective tissue as the principle, common source of tumors, an idea he would never fully give up. With the passage of time, Virchow's renown has obscured the historical details and largely deprived Remak of the credit due him. In Henry Harris'26 cogent metaphor, Remak was the discoverer whose voice was almost drowned in the publicity unleashed by Virchow, the colonizer.

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Helicobacter pylori and Ulcers

Richard G. Lynch, MD
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MILESTONES


These two landmark Lancet publications appeared almost 100 years after the first report of spiral bacteria in the human stomach and the initial speculation by several researchers that gastric ulceration was an infectious disease. Although more than 100 experimental studies suggesting a microbial cause of gastritis and peptic ulcers had been published in the first half of the 20th century, and several bacterial and viral species had been implicated as etiologic agents, the concept of an infectious pathogenesis for these common ailments was repeatedly rejected by influential authorities in both gastroenterology and pathology. The prevailing belief was that microbes could not survive in the acidic environment of the stomach and that bacteria present in resected stomachs and at autopsy were artifacts caused by contamination and post-mortem growth.

J. Robin Warren, a pathologist working at the Royal Perth Hospital in Western Australia, noticed curved rod-shaped bacilli in about half of the routine gastric biopsies he examined over a period of three years and found a direct correlation between the number of organisms in the tissue and the severity of gastritis. Convinced of the significance of his observations he enlisted the participation of Barry Marshall, a trainee in Internal Medicine, and a joint effort was launched to isolate the microorganism. Warren had noticed the resemblance of the curved bacilli to Campylobacter, a family of known intestinal pathogens. Using microaerophilic conditions that favor the laboratory growth of Campylobacter, they tried, unsuccessfully, to grow bacteria from stomach biopsies for more than a year. Serendipity delivered success when abundant bacterial growth was found in cultures that had been inadvertently left in the incubator over the Easter holidays, unintentionally extending the incubation period from two to six days.

While the isolation of Helicobacter pylori was a breakthrough achievement, it did not establish that the microbe caused gastritis. It was already known from autopsy studies that curved rod-shaped bacilli were present in the stomachs of many individuals who had neither gastritis nor a history of stomach disease. The successful isolation of bacteria from gastric biopsies by Marshall and Warren satisfied the first two of Koch’s four postulates, but all four had to be met to indisputably prove that the organism that had been isolated was the cause of the gastritis. In an amazingly daring feat that ultimately fulfilled Koch’s postulates, Marshall and another volunteer ingested cultures of the bacteria. Both of them developed acute gastritis proven by endoscopic biopsies from which the suspected pathogen was re-isolated. These results confirmed the link between H. pylori and gastritis, but since neither subject developed an ulcer, that link still remained unproven. Subsequent clinical trials showing that antimicrobial therapy could cure ulcers left no doubt that H. pylori caused gastric and duodenal ulcers. When Warren and Marshall used standard bacteriological tests and electron microscopy to characterize the isolated organism they found that it was not a Campylobacter species, but a newly discovered microbe that was subsequently designated Helicobacter pylori.

The findings of Warren and Marshall had enormous impacts. Peptic ulceration, a disease of world wide occurrence whose definitive treatment was surgical, became a disease that could be treated and cured with antibiotics. In the United States alone, approximately 4 million people have peptic ulcers. H. pylori infection is present in virtually all of them when the ulcer is located in the duodenum and in the vast majority of them when the ulcer is in the stomach. Once H. pylori could be cultured it became possible to determine the global prevalence and distribution of the infection. Immuno-epidemiologic tests to detect the presence of anti-H. pylori antibodies were performed on archived blood samples and
quickly established that at least 30-50% of the world’s population was colonized with *H. pylori*. Great variability was observed between different countries in the incidence of the infection and the age at which infection was acquired, and in the incidence of infection amongst different socioeconomic and ethnic groups. A surprising finding was that more than 80% of infected individuals were asymptomatic and only about one in six had an ulcer.

The milestone publications of Warren and Marshall triggered numerous basic and clinical investigations aimed at understanding the biology of *H. pylori*, the host response to encounter with the microbe, and the cellular and molecular mechanisms underlying the pathology of *H. pylori* infection. In a relatively short time these investigations proved to be extraordinarily productive and answered many of the questions that had previously fueled the skepticism surrounding the concept of an infectious etiology of ulcers. Numerous virulence factors encoded by the genes of *H. pylori* and passenger plasmids were identified and shown to influence colonization, persistence and pathogenicity of *H. pylori*. A high degree of genetic polymorphism in the expression of these virulence factors was observed in different isolates. This phenotypic heterogeneity provided insight into the highly variable host consequences of infection with *H. pylori*. The entrenched disbelief that microbes could survive in the strongly acidic environment of the gastric mucosa was annulled by the finding that *H. pylori* produced urease, an enzyme that made it exquisitely suited to survive in an acidic niche. Urea present in gastric secretions is cleaved by the microbial urease to yield ammonia and bicarbonate that create a moat of pH neutrality surrounding the bacterium. Urease activity became the basis of a clinical test that was developed to screen patients for the presence of gastric *H. pylori*. A major boost was given to the *H. pylori* field in 1997 when the entire DNA sequence of the bacterial genome was published in *Nature*.

Beyond its relevance to gastritis and ulcers, the discovery by Warren and Marshall ultimately led to the designation of *H. pylori* as a Class I carcinogen by the World Health Organization International Agency for Research in Cancer. Investigations that followed the landmark findings of Warren and Marshall established that the range of epithelial changes in chronic gastritis included hyperplasia, metaplasia, dysplasia and carcinoma. Decades before *H. pylori* had been isolated an association between cancer of the stomach and chronic gastritis had been recognized. The discovery that *H. pylori* was the major cause of chronic gastritis linked *H. pylori* and gastric cancer. In addition, pathologists had long observed a wide spectrum in the intensity of lymphoid cell infiltration and follicular development in stomachs with chronic gastritis. At times, distinguishing between chronic gastritis with intense lymphoid infiltration and gastric lymphoma could present a diagnostic challenge. In some *H. pylori*-infected patients the florid gastric lymphoid proliferation that was present met the diagnostic criteria of gastric lymphoma, but treatment of these patients with antimicrobial agents resulted in elimination of *H. pylori* and complete regression of the lymphoid proliferative process, an outcome that challenged the dogma that neoplasms are autonomous and neoplastic transformation is irreversible. Subsequent investigations showed that in some patients the neoplastic lymphoid cell proliferation was driven by host immune recognition of *H. pylori* antigens by lymphoid cells.

**References**


**Suggested Readings**

Plasmacytomases and Basic Immunology

Richard G. Lynch
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MILESTONES


These investigators reported the fortuitous discovery and initial characterization of plasmacytomases induced in BALB/c mice. Their findings launched an era of intense research and unprecedented discovery during which some of the most perplexing questions in immunology were definitively resolved. The structural basis of antibody specificity and the genetic basis of antibody diversity were established from investigations with murine plasmacytomases. The cornucopia of immunological information and the insights that were generated in research with plasmacytomases rightfully qualify them as immunological Rosetta Stones.

The initial and critical finding was by Merwin and Algire\(^1\) who implanted cell-impermeable diffusion chambers containing C3H mammary tumor tissue into the peritoneal cavity of BALB/c mice. A goal of the study was to determine if a virus that was present in the tumor, and associated with a high incidence of spontaneous breast cancer in C3H mice, would induce mammary tumors in BALB/c mice, a low incidence strain that did not carry the virus. None of the mice with diffusion chambers developed mammary tumors, but some of them developed peritoneal plasmacytomases\(^1\). This surprising finding raised the possibility that a virus could cause plasmacytomases. In a subsequent publication - a classic for re-emphasizing the need for controls in any experiment - Merwin and Redmon\(^1\) reported the surprising finding that plasmacytomases also developed in BALB/c mice implanted with empty diffusion chambers. It was soon recognized that a variety of substances induced peritoneal plasmacytomases in BALB/c mice. Potter and Boyce\(^1\) showed that simply injecting mineral oil into the peritoneal cavity of BALB/c mice induced plasmacytomases.

These studies appeared in the literature just as evidence from clinical investigations was suggesting that the so-called M-components, present in the sera of patients with certain autoimmune diseases, were actually homogeneous autoimmune antibodies. Until the discovery of murine plasmacytomases, efforts to investigate antibody structure using serum antibodies were limited by the heterogeneity of antibodies induced by immunization. Since almost every BALB/c plasmacytoma produced a serum M-component, these tumors presented a unique opportunity to investigate the structure and function of molecularly homogeneous immunoglobulins. Michael Potter and colleagues at the National Cancer Institute induced and characterized hundreds of different BALB/c plasmacytomases and generously made them available to investigators throughout the world.

Immunochemists were immediately attracted to plasmacytomases because they provided essentially an unlimited source of homogeneous immunoglobulins. The monoclonal proteins produced by most of the BALB/c plasmacytomases were IgA or IgG. Some produced only a kappa or lambda light chain, and rare plasmacytomases produced IgM or IgD. The amino acid sequences of purified heavy and light chains from dozens of the plasmacytomases indicated that most of them produced a unique monoclonal immunoglobulin. The pooled structural information immediately generated insight into the organization of the genes that encoded antibodies. The amino acid sequence data identified constant and variable regions, hypervariable regions, heavy chain domains, classes and subclasses, hinge regions, allotypic markers, disulfide loops, and a wealth of other information that established most of our current understanding of antibody structure.

A longstanding quest of immunologists was to understand the structural basis of antigen recognition by antibody molecules. The availability of hundreds of different plasmacytomases made it feasible to attempt such structure-function studies. A number of investigators began screening Potter's library of monoclonal immunoglobulins hoping to find some with antibody activity. A considerable number were found to bind conventional haptens, such as 2,4-dinitrophenyl (DNP) and

Milestones in Investigative Pathology, Richard G. Lynch, MD
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phosphorylcholine, and some bound carbohydrate antigens. Affinity-labeling reagents began to pinpoint the antigen binding sites. Quantitative and equilibrium analyses of hapten binding provided information about valency and binding kinetics of individual antibody molecules. Eisen and colleagues extensively characterized the DNP-binding IgA proteins produced by plasmacytomas. Subsequent research in several laboratories visualized the stereostructure of antibody combining sites.

In a series of elegant studies Coffino, Laskov and Scharff used plasmacytoma cell culture and single cell cloning in soft agar to investigate the molecular details of immunoglobulin production. They identified the steps in synthesis, assembly and secretion of antibody molecules, and the role of somatic mutation in the generation of variant cells that only produced a clonal light chain. Non-producer variants would later prove important in the development of hybridomas.

A number of investigators examined pathological and immunobiological aspects of murine plasmacytomas. Zolla-Pazner characterized the mechanisms of the immunodeficiency that occurs in mice bearing plasmacytomas, a condition that mimics the immunodeficiency in patients with multiple myeloma. Those studies showed that plasmacytoma cells elaborate immunological signals that influence the host's immune system. The converse was proven in studies by Lynch and colleagues that showed the growth and differentiation of plasmacytoma cells could be regulated by specific immunological signals programmed into the host by prior immunization.

In the ingenious hybridoma system developed by Kohler and Milstein, murine plasmacytoma cells were fused with immune spleen cells to immortalize clones that produced essentially unlimited quantities of a single antibody of defined specificity. Kohler and Milstein shared a Nobel Prize in 1984 for their hybridoma work, the impact of which continues to resonate widely across the biological sciences, human medicine and beyond.

The investigators who initially used plasmacytomas to study antibody structure studied the proteins directly. With the advent of molecular genetic technology, the focus shifted to the analysis of antibody genes. In the mid-1960's observations of the inheritance patterns of human immunoglobulin allotypes suggested that each immunoglobulin chain was encoded by two distinct genes. This idea violated the established dogma that each polypeptide chain was encoded by a single gene. The two gene hypothesis further complicated the central immunologic puzzle of the day: how was the extraordinary diversity of antibodies generated?

The answers came from the research of Susumo Tonegawa, the pioneer in applying recombinant technology in immunology. He was awarded the Nobel Prize in 1987 for his ground-breaking discoveries. Plasmacytomas were a critical element in the design of the research. Tonegawa's research team prepared and fractionated restriction enzyme digests of DNA from a kappa light chain-producing plasmacytoma and from mouse embryo DNA. Using kappa constant and variable region hybridization probes, they found that in the embryonic DNA the kappa constant and variable region gene segments were separated from each other by considerable distances, but in the plasmacytoma DNA they were joined to form a much smaller, contiguous stretch of DNA. This indicated that rearrangement of the DNA took place when a cell produced a light chain. Further studies of kappa light chain DNA from a library of plasmacytomas showed that kappa light chains are encoded by a gene that is formed by combining one constant region gene segment with one of the approximately 250 variable region gene segments present on the same chromosome. Other studies showed that the heavy chain gene was formed by combining a single constant region gene segment with one of the approximately 800 heavy chain variable region gene segments present on the same chromosome.

As many laboratories pursued the study of immunoglobulin genes using murine plasmacytoma cells, it became clear that, in addition to the constant and variable region gene segments, there were other, very small gene segments that were rearranged and incorporated into the functional gene. These additional gene segments, one for light chains and two for heavy chains, were selected from a large number present on the respective chromosome. The process of combining these gene segments into the functional gene was found to be error prone at the splicing sites. This resulted in small changes in the DNA sequences at the joints.

The significance of all this complexity is that it explains how a repertoire of at least 10^9 different antibody molecules can be generated from approximately 10^3 gene segments. The possible gene segment combinations, coupled with the splicing site errors, yields an extraordinary number of functional genes. Even before BALB/c plasmacytomas were discovered, it was estimated that the size of the antibody repertoire was in the range of 10^8. If the dogma of "one polypeptide chain: one gene" was correct, then there was insufficient DNA in the genome for 10^9 antibody genes. Plasmacytomas provided researchers with the experimental system that solved this longstanding immunologic conundrum. It is currently estimated that the mouse genome contains about 3 x 10^6 genes.

The tremendous advances that came from research with plasmacytomas showed that tumor cells can provide powerful research tools for understanding the processes of normal cells. This principle has been extended to investigations of other lineages of immune cells using other types of lymphoid tumors.

The straightforward, descriptive research described in the two Milestone articles spawned a profusion of basic knowledge that has advanced science and benefited society. The decisions to conduct the initial studies, and to provide resources to support them, can be seen through the "retrospectiscope" as tremendously wise. There might be some lessons here.

References
Until these meticulous studies by Paris Constantinides were published, the pathogenesis of arterial thrombosis was a subject of considerable controversy and speculation. It had long been known that thrombotic occlusion of a coronary artery was a common autopsy finding in patients dying from myocardial infarction (heart attack), and that cerebral artery blockage by a recently formed clot was a typical finding in patients with a cerebral infarction (stroke). While there was strong consensus that advanced atherosclerosis predisposed coronary and cerebral arteries to thrombosis, the mechanisms underlying these events had remained obscure.

The hallmark lesion of atherosclerosis is the atheromatous plaque, or atheroma, a term derived from the Greek word for gruel. An atheroma is a raised focal plaque within the arterial intima that is composed of a pasty, cholesterol-rich core covered by a fibrous cap. With time, atheromas become calcified and acquire the brittleness of an egg shell.

In a minority of patients with coronary or cerebral thrombosis, the clot forms over an ulcerated atherosclerotic plaque. This was readily explained because the collagen, calcium and phospholipoproteins exposed in a denuded plaque were well known aggregators of platelets and activators of thromboplastin. However, in the majority of patients with coronary or cerebral thrombosis, the clot formed over seemingly intact, hemorrhagic plaques. The pathogenesis of these clots remained unexplained until the milestone investigations of Constantinides.

Several mechanisms had been proposed to account for coronary and cerebral thrombosis, but each was conceptual, speculative and unproven. The consistency of finding hemorrhage within seemingly intact plaques at the site of thrombosis spawned a novel concept – the capillary hemorrhage theory, which proposed that capillaries from the arterial lumen invaded the plaque and then ruptured, triggering a retrograde thrombosis that expanded to occlude the artery. This theory ignored the fact that capillary invasion of plaques usually occurs from the adventitia – the outside of the artery – not from the lumen.

The stasis theory proposed that diminished blood flow caused by atherosclerotic narrowing of the vessel resulted in thrombus formation, while the turbulence theory envisioned thrombosis as the result of eddying-induced platelet aggregation at sites of atherosclerotic narrowing. The enormous morbidities and mortalities associated with coronary and cerebral thrombosis, disorders highly prevalent in developed nations, fostered major research efforts in North America and Europe. However, progress was stymied by the lack of a relevant experimental model and by a reductionist research approach to the overwhelmingly complex process of atherogenesis. Although the list of predisposing conditions, such as hypertension, hyperlipidemia, diabetes, obesity and cigarette smoking, continued to grow, real progress towards identifying pathogenic mechanisms proved elusive.

A popular concept held that thrombus formation in an atherosclerotic coronary or cerebral artery was a result of systemic hypercoagulability. Support for this idea came from clinical trials in which anticoagulants appeared to improve survival in selected patients. A major criticism of the hypercoagulability theory was its failure to account for the exclusive formation of only a single clot, at a single site, in a single artery, in spite of numerous atherosclerotic lesions present in the same and other arteries of the patient.

The tiny fissure hypothesis of Constantinides proposed that the thrombi that form over seemingly intact hemorrhagic plaques are caused by microscopic cracks in the collagenous caps of the plaques – cracks so small that they are rarely detected by routine histological examination. To test his idea, Constantinides conducted a monumental
examination of serial seven-micron-thick paraffin sections cut through the entire length of occluded coronary arteries. This meticulous study of thrombosed segments from 20 consecutive autopsy cases of coronary thrombosis involved more than 40,000 microscopic sections!

Constantinides observed that in every case the thrombi were anchored in fissures of the caps of atherosclerotic plaques. The fissures were about 300 to 400 microns in length, a size where fissures would likely be missed if one or only a few random histological sections of the thrombus were examined. The fissures tended to occur at the margins of plaques, where the edge of the cap attached to the uninvolved arterial wall. The adherence of the thrombus to the fissure occurred in the platelet-rich zone of the thrombus, the region where clotting is initiated. He concluded that almost all plaque hemorrhages were caused by tiny cracks in the cap that triggered thrombus formation when blood came in contact with the thrombogenic material contained inside of plaques.

In another massive undertaking, Constantinides employed the same approach to examine 10 consecutive autopsy cases of cerebral artery thrombosis. He observed that all thrombi were attached by their platelet-rich zones to tiny cracks in the caps of atherosclerotic plaques.

Subsequent studies by other investigators confirmed the observations of Constantinides, and it became accepted that thrombosis in atherosclerotic coronary and cerebral arteries is practically always initiated by local breaks in the surface of atherosclerotic plaques. By the early 1960's a rabbit model of human atherosclerosis had been developed. Using this model Constantinides showed that the induction of breaks in the experimentally induced atherosclerotic plaques resulted in the formation of overlying thrombi. In rabbits with experimental atherosclerosis, but not in control rabbits, an induced burst of hypertension caused plaque fissures and overlying thrombosis.

An interesting characteristic of scientific progress is the frequency with which competing hypotheses are eventually found to contain valid elements. In the instance of arterial thrombosis research, there are elements of truth in most of the hypotheses that had been put forward. This likely reflects both the complexity of atherogenesis and the multiplicity of its complications.

Hypercoagulability remains an important detail of coronary and cerebral thrombosis and underlies the recommendation of daily intake of small doses of aspirin for individuals at risk for myocardial infarction or strokes. Where the hypercoagulability theory envisioned a systemic clotting disorder, Constantinides' findings pointed to localized clotting that occurred when a fissure allowed blood to come in contact with thrombogenic materials contained within a plaque.

Constantinides later suggested that plaque fissures might occur all the time, but the rate at which thrombi grow, the size they attain, and their clinical consequences might depend on systemic factors, such as hypercoagulability and fibrinolysis, and local factors, such as stasis and turbulence. He further proposed that mechanical factors, such as bursts of hypertension, or the constant bending and torsion of coronary arteries with each ventricular contraction, could result in the formation of cracks in brittle atherosclerotic plaques.

The milestone research of Constantinides is a sterling example of the vital role of the individual investigator in advancing biomedical knowledge. The research enterprise of Paris Constantinides consisted of himself, who conducted some of the autopsies, a histology technician, the secretary who typed the manuscripts, and colleagues who provided some of the specimens. The research was supported by a small NIH grant.

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Lymphocyte Traffic

Richard G. Lynch, MD


MILESTONES


In a landmark publication, Gowans and Knight at the Dunn School of Pathology in Oxford reported that the output of lymphocytes from the thoracic duct of rats (about 10^9/day) is normally maintained by a large scale recirculation of cells from blood to lymph. The studies were made possible by skilled investigators who mastered the technique of thoracic duct cannulation. The thoracic duct, the terminal lymphatic draining conduit that connects the lymphatic and blood vascular systems, empties its lymphoid cells and protein-rich fluid into the superior vena cava. It was known that chronic cannulation of the thoracic duct created a lymphopenic state, suggesting that after entering the blood, the cells from the thoracic duct reenter the lymphatic system.

The studies from Gowan’s laboratory established that the traffic of lymphocytes between the lymphatic and blood vascular systems was highly selective, not random. This implied that the process involved specific recognition and interaction between molecules on the surface of small lymphocytes and complementary molecules expressed on the surface of the endothelial cells of high endothelial venules (HEVs) in lymph nodes. The finding that large lymphocytes from the thoracic duct emigrated to the intestine and not to lymph nodes suggested that different populations of lymphocytes emigrated to different anatomical sites, a concept that today is well established.

The tool that revolutionized the study of lymphocyte migration was described in the sentinel publication of Stamper and Woodruff. They incubated thoracic duct lymphocytes (TDL) with fixed histological sections of rat lymph nodes and observed highly selective adherence of small lymphocytes to the endothelium of HEVs. This in vitro assay correlated precisely with earlier in vivo studies that showed small lymphocytes migrated from blood into lymph nodes only via HCVs, and that the small lymphocytes from rat thymus and bone marrow rarely bound to lymph node HEVs.

With the specific and reproducible in vitro assay developed in the Woodruff laboratory it became possible to investigate lymphocyte binding to...
HEVs at a mechanistic level. In a breakthrough set of studies, Gallatin and Butcher and their mentor Irving Weissman at Stanford developed a monoclonal antibody that recognized a surface molecule on lymphocytes involved in organ-specific homing\(^1\). By immunizing rats with a clone of mouse lymphoma cells that bound to lymph node HEVs they produced a monoclonal antibody (MEL-14) that blocked the \textit{in vitro} binding of small lymphocytes to lymph node HEVs but not to Peyer's Patch HEVs, and blocked the homing of normal lymphocytes \textit{in vivo}. MEL-14 allowed for preliminary isolation and characterization of the lymphocyte surface molecules involved in binding to lymph node HEVs.

The Stanford investigators blazed the pathways of lymphoid cell traffic, an area of investigation that attracted numerous other investigators, broadened its focus and has been extraordinarily productive of new knowledge. A growing list of chemokines, chemokine receptors and tissue-specific adhesion molecules have been discovered, characterized and shown to coordinate cell migration. The expression of unique combinations of adhesion molecules and chemokines have been shown to underlie tissue-specific migration, such as the homing of IgA+ B cells to the mammary gland late in pregnancy and during lactation\(^5\). The cytokines and other factors that regulate the expression of these combinations continue to be identified\(^6\).

When the studies of Knight and Gowans were published in 1964 the field of immunology was still focused at the level of humoral immunity: the field of cellular immunology had just started to emerge from the dark ages. The heterogeneity of lymphoid cell lineages and functions were just being discovered. Previously it was thought by some that the function of small lymphocytes was trophic. It was reasoned that since lymphocytes circulated widely, had almost no cytoplasm, and were loaded with DNA, they functioned to bring DNA to cells throughout the body, like mobile fueling stations delivering DNA to cells in need of a fill up.

The milestone publication by Knight and Gowans was a pivotal step in the emergence of cellular immunology, and by the time the publication of Stamper and Woodruff appeared, the field was moving in high gear. Today we know a great deal about the genes, molecules, regulatory systems and mechanisms involved in the targeted movement of lymphocytes. We know that it is a mechanism for dispersing the immunologic repertoire, for directing lymphocyte subsets to the specialized microenvironments that control their differentiation, for regulating lymphocyte survival, and for targeting immune effector cells to the sites of antigenic or microbial invasion. Characteristic patterns of lymphocyte traffic underlie the organization of regional immunity, such as mucosal, cutaneous and reproductive immune responses.

As the molecular basis of lymphocyte traffic expanded, it became evident that similar systems and families of molecules operate in the traffic of other leukocytes, and might function in the movements of neoplastic and embryonic cells. As the complex molecular mechanisms involved in cellular traffic become fully understood, it could provide the knowledge to construct strategies that therapeutically manipulate normal and pathologic cellular migration.

References
If you examine a list of publications having unusually high impact in a scientific field, close to the top will often be a breakthrough article on methodology. The ground-breaking report by Richard Farr is an excellent example of this principle. At the time Farr's paper appeared, assays in use did not quantitate the amount of specific antibody present in a serum sample. They measured some activity that was a consequence of antibody binding to antigen. The level of that activity in a tested sample was expressed as its titer, usually the dilution beyond which the activity could no longer be detected.

Some of the consequences of antibody binding to antigen were measured in vivo, such as by Arthus reactions, anaphylaxis, or neutralization of a toxin or pathogen; others were measured in vitro with readouts such as complement fixation, precipitation, hemolysis or agglutination reactions. The specific hapten-binding capacity of an immune serum could be measured by equilibrium dialysis but that assay did not quantitate antibody.

Farr's classic paper appeared a decade before the start of the explosive growth of knowledge about antibody structure and function. It remained to be discovered that the various activities being titered were mediated by different classes and subclasses of antibodies. Immunochemists knew that some antigens scoring as weak inducers of antibody responses in one assay sometimes scored as strong inducers in a different assay and that the assays were not interchangeable. They recognized the curious properties of non-precipitating and blocking antibodies, and appreciated that most assays detected only some of the antibodies present in the sample tested. The lack of an assay that quantitated the primary interaction between antibody and antigen formed a serious gap in the tools available to immunochemists.

The Farr Assay became the first laboratory test to fill that void. The beauty of the Farr Assay resided in its ability to measure the primary interaction between antigen and antibody. It did not require the additional step of producing some functional activity such as precipitation or complement fixation.

Farr made the important discovery that at low concentrations, immune complexes could be separated from unbound antigen by differential solubility in concentrated solutions of ammonium sulfate. He observed that when bovine serum albumin (BSA) – trace labeled with radioactive iodine – was mixed at excess with diluted samples of anti-BSA antibody, the BSA:anti-BSA immune complexes – but not the unbound BSA – precipitated when the mixture was 50% saturated with ammonium sulfate. The assay provided a method to detect all the antibodies in the sample that combined with BSA to form immune complexes.

Farr's breakthrough paper reported more than a new assay. It was a tour-de-force of experiments and controls directed at understanding the interaction between antigen and antibody. Farr and his colleagues later adapted the assay to quantitate antibodies specific for ragweed antigen, Streptococcal M protein and endotoxin. Other antigens followed.

The principle of the Farr Assay – separating bound from unbound antigen – revolutionized the measurement of antibody responses. The assay detected immune complexes, the first step in antigen:antibody interactions. Farr's discovery launched the field of immunoassays, which as it grew led to numerous applications in clinical and research laboratories. Today, hundreds of immunoassays are routinely used for diagnosing diseases and monitoring responses to therapy. As the field of immunochemistry progressed, many investigators found clever new ways to detect antigen:antibody complexes. Some assays detected immune complexes using labeled antigen, some used labeled antibody, and others employed indirect methods.

Over the years, concerns about handling and disposing of radioactive materials led to the development of novel, non-radioactive readouts. Enzymatic production of color by immune complex:enzyme conjugates began to
replace the measurement of radioactivity present in antigen:antibody complexes.

The configuration of the Farr Assay made it a simple matter to measure competitive binding of labeled antigen by unlabeled antigen in a sample. Reference to standard curves allowed precise quantitation of the amount of antigen in a tested sample. The development of competitive radioimmunoassays rapidly led to their widespread use in the analysis of physiological fluids. It became possible to rapidly quantitate serum levels of drugs such as digoxin, and biologic mediators such as peptide hormones. While many derivative assays have replaced the original Farr Assay in routine laboratory testing, when comparisons are made, the Farr Assay consistently proves to be more sensitive. In a recent study, 173/178 serum samples from patients with Chagas disease were positive for Chagas-specific antibody by ELISA. By Farr Assay, 177 of the 178 samples were positive. These findings have implications for the safety of blood transfusions. Studies of autoantibodies specific for double-stranded DNA and autoantibodies directed to laminin-5 confirm the superior sensitivity of the Farr Assay. In laboratory testing for compounds present at extremely low concentrations, the Farr Assay still plays an important role.

Richard Farr made his breakthrough discovery fairly early in his research career. He subsequently went on to a long, productive and distinguished career as a physician-scientist at the National Jewish Hospital in Denver.

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Hypertension and the Kidney
Richard G. Lynch, MD
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MILESTONES

This landmark publication by Harry Goldblatt1 and his colleagues at the Institute of Pathology at Western Reserve University in Cleveland revolutionized ideas about the pathogenesis of hypertension.

Credit for making the connection between hypertension and renal disease is usually given to Richard Bright, a physician at Guy's Hospital in London even though he worked in the early 1800's, a half century before the existence of hypertension was first recognized.

Bright observed that large, heavy hearts found at autopsy, in the absence of other explanations, often occurred in patients with abnormal kidneys. He exhibited incredible insight by suggesting that a blood chemical of renal origin might be the cause of the enlarged hearts. He speculated that heart enlargement reflected an elevated cardiac workload due to increased peripheral resistance. Although he never measured a blood pressure, in view of what we know today about hypertension and the kidney, Bright was pretty much on target.

Prior to Goldblatt's publication, a large body of experimental work had tested the idea that hypertension had a renal basis. The approaches used had the common outcome of impairing renal excretory function. They included variable degrees of renal ablation; renal vein constriction; and permanent bilateral ligation of the renal artery, renal vein and ureter. These manipulations sometimes resulted in a fleeting elevation of blood pressure, but usually did not.

Goldblatt took the approach of experimentally compromising renal arterial blood flow by placing a clamp on the main renal artery. He got the idea from the observation well-known to pathologists that intrarenal sclerosis of arteries and arterioles were commonly found at autopsy in patients dying with hypertension. Recognizing that no experimental procedure existed for creating the vascular pathology seen in human hypertensive kidneys, he reasoned that if impaired renal blood flow was the fundamental cause, this could be mimicked by constricting the main renal artery.

Silver clips, especially fabricated for these experiments, were set for varying degrees of constriction and placed on the renal artery of dogs. Goldblatt observed that mild constriction of the main renal artery was sufficient to induce a rise in blood pressure within 24 to 72 hours. In control experiments constriction of the splenic or femoral arteries did not result in elevated blood pressure.

Once hypertension was established, removal of the clip resulted in return of blood pressure to normal levels, a finding suggesting that the ischemic kidney maintained the elevated blood pressure. In some experiments, instead of removing the clip, the clipped kidney was removed. This resulted in a return of blood pressure to normal levels. Subsequently placing a clip on the main renal artery of the remaining kidney resulted in reelevation of blood pressure.

In Goldblatt's early studies, hypertension in most animals lasted from 4 to 6 weeks and then blood pressures returned to normal levels, even though the clamps were still in place. An astute anatomical pathologist, Goldblatt noticed that the return to normal blood pressure was associated with conspicuous development of collateral arterial circulation to the kidney, particularly through the renal capsule. In subsequent experiments he decapsulated the kidney and enclosed it in a membrane to prevent revascularization. When the renal artery of such animals was constricted, hypertension occurred and persisted.

Goldblatt's discovery was soon followed by similar experiments by other investigators using sheep, goats and rats. Interestingly, some argued that Goldblatt's model had little relevance to human hypertension because of the belief, subsequently shown to be erroneous, that renal artery stenosis rarely occurred in humans. It took careful autopsy observation and the development of
sophisticated radiological imaging of arterial vasculature to establish that in 2-5% of cases of human hypertension, patients have a “Goldblatt kidney.”

In 70% of these patients renal artery narrowing is due to an atherosclerotic plaque. In the other 30%, typically young females, the narrowing is due to fibromuscular dysplasia of the renal artery. A critical feature of these lesions is that they are correctable. Considering the incidence of hypertension in the general population, hypertension caused by renal artery narrowing is not an uncommon disease.

The experiments of Goldblatt and colleagues further buoyed the idea that the kidney produced a chemical substance that elevates blood pressure. Their findings launched studies by investigators around the world. These led to the purification and characterization of renin; identification of the juxtaglomerular apparatus as its site of synthesis; and biochemical characterization of renin's target effects on the angiotensin system, blood pressure and aldosterone secretion by cells in the adrenal cortex.

Three quarters of a century of physiology and pharmacology research flowed from the Goldblatt discovery, research that led to extraordinary advances. The clock is still ticking. Their classic paper is another example of the tremendous impact that observations made at autopsy have in posing critical questions and guiding the design of laboratory experiments that elucidate disease pathogenesis. The history of medical advances is replete with examples.

It is mind boggling that today's physicians, including many pathologists, seem to have forgotten the incredible power of the autopsy in advancing public health. Tragically, all the evidence I have seen suggests that this memory deficit results from system-wide attitudinal lesions that may not be reversible.

Reference

The Neural Crest and Neurocristopathies

Richard G. Lynch, MD
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The neural crest, initially described by Wilhelm His in 1868 as a novel longitudinal band of cells dorsal to the spinal cord, is a fascinating and unique progenitor tissue. Fascinating because it gives rise to a surprising diversity of specialized cells and tissues. Unique because it is an embryonic germ cell structure that disappears.

Abnormalities of neural crest development result in a bewildering myriad of diseases, some involving complex syndromes of seemingly unrelated lesions that until recently defied any unifying explanation. Groundbreaking investigations by the French embryologist Nicole Le Douarin launched an era of unprecedented discovery in developmental biology. The impact of those discoveries continues to enrich our understanding of developmental diseases and their pathogenesis.

Research during the first half of the 20th century, mostly studies of lower vertebrates, established that neurocrest cells delaminate from the primitive neural folds early in embryonic life and coalesce above the neural tube to form the neural crest. Le Douarin and colleagues, using microsurgical techniques, removed the neural folds from chick embryos before the onset of migration and replaced them with neural folds from quail embryos matched for developmental stage. Exploiting the biological marker in quail cells, they showed that virtually every tissue in the embryonic and adult body contains cells originating in the highly invasive and pluripotent neural crest.

In a series of investigations Le Douarin and colleagues extirpated segments of chick neural crest and replaced them with the corresponding quail segments. This approach allowed them to map the destination of neural crest cells at different locations along the neural crest. Their experiments established that the axial level of origin in the neural crest determined the migratory pathways taken.

Four major regions of the neural crest were recognized: cephalic, vagal, truncal and sacral.

Le Douarin’s group established that cephalic neural crest cells formed most of the bones of...
the head and facial skeleton. They also formed the connective tissue associated with striated muscles of the head and neck, the buccal and pharyngeal glands, the parathyroid glands, the thymus, and the conotruncal region of the heart. These findings would later provide strong clues about the pathogenesis of complex developmental disorders, such as DiGeorge syndrome.

Humans with DiGeorge syndrome exhibit agenesis or hypoplasia of the thymus and parathyroids, conotruncal abnormalities of the heart, and craniofacial dysmorphism. Each of these lesions occurs in tissues derived from cephalic neural crest. Experimental ablation of the cephalic neural crest in chick embryos results in an identical combination of defects.

DiGeorge syndrome maps to a locus on chromosome 22 that encodes a protein with the structural features of a transcriptional regulator. Mice homozygous for a targeted null mutation of the homologue have defects in multiple cranial and cardiac neural crest derivatives, including the cranial ganglia, aortic arch arteries, cardiac outflow tract, thymus, parathyroid glands and craniofacial structures.

The Le Douarin group showed that cells from the vagal crest colonized the gut to form the enteric nervous system.

They also showed that the truncal crest gave rise to pigment cells of the skin, spinal dorsal root ganglia, paravertebral sympathetic ganglia, Schwann cells of all peripheral nerves, the adrenal medulla and paraganglia. The sacral crest contributed to the innervation of the distal hindgut.

Some neurocristopathies occur as tumors, others as malformations; some as isolated lesions, others as combinations of lesions clustered in a syndromic pattern. Isolated and combinatorial lesions occur in both sporadic and familial forms.

Tumors derived from neural crest cells include neurofibroma, neuroblastoma, medullary thyroid cancer, pheochromocytoma, melanoma and several less common neoplasms.

Neural crest malformations include aganglionic megacolon (Hirschsprung's disease), congenital nevi, albinism, cleft lip and/or palate, conotruncal heart malformations and the lesions in fetal alcohol syndrome.

Our current knowledge of neural crest ontogeny, the genetic and microenvironmental factors that govern its properties, and the developmental defects that give rise to its disorders all trace their pedigree to the milestone research of Nicole Le Douarin. Reviews of these advances have been published.

The tools of molecular biology made labeling of the neural crest in mice a reality. Neural crest-derived cells are readily identified in transgenic mice in which the expression of beta-galactosidase or green fluorescent protein is driven by promoters of genes expressed in neural crest cells. Permanent long-term labeling occurs in double transgenic mice in which the production of Cre recombinase is conditioned upon expression of either Wnt1 or Sox10 genes.

Genetic studies continue to identify pivotal mutations that clarify the previously baffling combination of pathologic findings in syndromic neurocristopathies. Mutations in the receptor tyrosine kinase RET and the endothelin-β receptor underlie two familial forms of aganglionic megacolon (Hirschsprung's disease). Targeted disruption of the mouse endothelin-3 ligand results in megacolon and pigment disorders, the latter also seen in some patients with Hirschsprung’s Disease and a hallmark of Von Recklinghausen's disease. Some Hirschsprung patients develop neuroblastomas or other neoplastic forms of neurocristopathy. All of these combinations have in common a linkage with vagal neural crest.

Neurofibromatosis Type 1 (NF1 gene) is one of the most common genetic diseases and neurocristopathies. Affected individuals develop a multiplicity of cutaneous and visceral neurofibromas, pigmented lesions of the skin, and one or more pigmented iris hamartomas. Some patients develop adrenal gland pheochromatocytomas. At first glance these seem esoteric combinations of lesions, but when considered in terms of neural crest ontogeny, the relationships of the lesions become obvious.

The impact of Le Douarin’s research continues to grow with recent findings showing that environmental agents can cause neurocristopathic effects in humans. The frequent occurrence of non-familial neurocristopathies suggested that environmental factors played a causal role. Several studies have now shown that the consumption of alcohol during early pregnancy and the dermatological use of 13-cis-retinoic acid for the treatment of severe acne in pregnant women produce characteristic malformations in neural crest-derived tissues.

Le Douarin took a simple observation about quail nucleoli and transformed it into a brilliant discovery. That leap forward resonates with the definition of discovery suggested by the Hungarian physiologist and Nobel laureate Albert Szent-Georgy: "...seeing what others have seen, but thinking what others have not."

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Pneumococcal Transformation: Genes are Made of DNA

Richard G. Lynch, MD
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MILESTONES

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The finding that DNA transmits heredity is considered by many to be the most important discovery in biology during the 20th century. Amazingly, the reception given the publication by Avery and colleagues announcing their discovery hardly fit such a fundamental advance. To a surprising degree the publication went unnoticed. The article appeared at the height of the Second World War — a time of hampered communication within the international scientific community — and in a journal not considered a main line periodical for geneticists and general biologists. The paper by Oswald Avery, Colin MacLeod and Maclyn McCarty did catch the attention of some biochemists who generally greeted it with skepticism. The claim that genes are made of DNA challenged the established dogma that proteins were the carriers of genetic information. Almost a decade passed before the discovery by Avery and his colleagues became widely known and acknowledged as a sentinel event in genetics.

The history of the ground-breaking discovery by Avery, MacLeod and McCarty is a fascinating story filled with valuable lessons for young investigators and for the institutions that provide them the resources needed to do research. Maclyn McCarty elegantly detailed that history in a book that allows the reader to vividly experience the daily elations and disappointments of life at the laboratory bench during the first half of the 20th century. Avery, the senior investigator, and his trainees MacLeod and McCarty were medical bacteriologists at the Hospital of the Rockefeller Institute in Manhattan. Avery’s research focused on the pathogenesis of pneumococcal pneumonia, at the time the leading cause of death in the United States, far surpassing deaths due to heart disease and cancer. His ultimate goal of eradicating pneumococcal pneumonia always eluded him, but in the process of pursuing it for three decades he and his colleagues discovered the chemical basis of heredity.

When Avery began his work at Rockefeller in 1913, the fundamental properties of pneumococci had already been established. Pasteur had initially isolated the virulent microbe from the sputum of a patient with pneumonia. Later investigators described the organism's thick capsule and observed that a single pneumococcus injected into a mouse proved lethal. Rabbits and horses immunized with pneumococci from one patient developed antibodies that reacted with the capsule of that microbe and with the capsule of pneumococci isolated from some, but not all, other patients. The anti-capsular antibodies defined four serological types of pneumococci (I, II, III, IV). Several laboratories had shown that type-specific antibodies from rabbits could protect mice from an otherwise lethal challenge with pneumococci of the corresponding type.

Shortly after his appointment at Rockefeller, Avery discovered that the supernates of pneumococcal cultures contained a soluble component precipitated by type-specific antibodies. Michael Heidelberger, a young colleague at Rockefeller, showed that the soluble component Avery had termed "soluble specific substance" was a polysaccharide. Evidence developed that the soluble polysaccharide came from the capsule of pneumococci and that the capsule was critical to the organism's virulence. Proof of the latter came from the discovery that pneumococci cultured in the presence of type-specific antibodies became nonencapsulated and totally avirulent. Mice showed no ill effect when injected with billions of unencapsulated pneumococci, but succumbed to injection of a single encapsulated microbe. Microscopic studies revealed that blood phagocytes rapidly ingested nonencapsulated pneumococci, but not the virulent encapsulated forms. However, when type-specific antibodies were present, blood phagocytes readily ingested the encapsulated pneumococci. The effect of antibody on pneumococcal virulence reinforced the rationale for using immune horse serum to treat patients with pneumococcal pneumonia.

Martin Dawson, a young associate in Avery's laboratory, initiated studies to determine if non-
encapsulated, avirulent pneumococci could undergo reversion to the encapsulated, virulent form. He found evidence for reversion and published his initial findings in 1928. This launched 15 years of experiments of bewildering complexity and erratic reproducibility by Avery and his colleagues. Ultimately, they identified the mechanism of reversion and in the process discovered that genes are made of DNA. In their milestone publication they reported that when non-avirulent pneumococci isolated from Strain II were cultured in the presence of DNA from virulent type III pneumococci, the Strain II microbes produced Type III capsular polysaccharide and became virulent.

Avery’s findings evoked strong skepticism from several prominent biochemists who argued that protein contaminants in the DNA likely accounted for the transformation from Type II to Type III. At the time it was well established that enzymes, antibodies and other proteins exhibited high specificity, characteristics favoring their role as informational molecules. Furthermore, based on evidence at best vague but unchallenged, most biochemists considered nucleic acids totally unsuited to contain genetic information. The prevailing concept envisioned nucleic acids as monotonously similar small molecules composed of stacks of flat tetranucleotide rings that functioned to hold the chromosome together.

Avery’s group went to great lengths to rule out protein contamination of their DNA preparations. Using the most sensitive analytical methods then available, they failed to detect protein in their preparations. The transforming activity in their material totally resisted the action of proteolytic enzymes and methods known to denature proteins. The recovery of transforming activity from transformed cells in amounts far in excess of that originally used to induce transformation implied replication of the transforming principle. Purified preparations of DNase did not exist so McCarty spent two years developing a procedure to isolate DNase from bovine pancreas. In a 1946 paper he reported that the transforming activity was destroyed by DNase.

In spite of all the evidence presented by Avery and his colleagues that genes are made of DNA, their work continued to be criticized or ignored for almost a decade after their milestone publication. Geneticists expressed doubts about the relevance of findings in bacteria to hereditary mechanisms in higher organisms. In spite of the generally dubious reception the 1944 paper received, it did have an impact. It clearly influenced the eminent biochemist Erwin Chargaff to undertake analysis of DNA from different species, studies that eliminated the prevailing notion that nucleic acids are all alike. As he pursued those studies Chargaff discovered A-T and G-C base pairing, a crucial determinant in the DNA model proposed by Watson and Crick. The 1944 paper also directly influenced the thinking of Francis Crick as he and Watson began their work. Commenting on the discovery by Avery, MacCloud and McCarty forty years later, Nobel laureate Joshua Lederberg ranked its importance with the contributions of Darwin and Mendel.

It is unlikely that in today's research climate Avery's laboratory could have sustained the decades of effort that led to the groundbreaking discovery. There were long periods of slow progress, negative findings and few publications. It is often pointed out that knowledge gained through basic research advances the understanding and treatment of disease. There are fewer reminders that knowledge gained by investigating disease can advance our understanding of basic biological processes. The pneumococcal research of Avery and colleagues is a splendid example of the latter principle.

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Virtually anyone who has ever grown mammalian cells in culture has encountered the term Eagle's medium. But only some of them will know much about the medium's formulator, Harry Eagle. In his 1955 *Science* paper, Eagle summarized his extensive investigations that identified a chemically-defined medium that supported the *in vitro* growth of two cell lines, one a mouse fibroblast, the other a human carcinoma. Eagle's studies proved to be a tremendous breakthrough in the field of *in vitro* culture of mammalian cells.

Prior to Eagle's pioneering studies, the growth of mammalian cells *in vitro* involved explants of tissue pieces, a biological matrix such as a plasma or fibrinogen clot where the growing cells attached, and a liquid medium composed of human placental serum or adult serum, chicken embryonic extract and a balanced salt solution. Usually the cells grew for variable lengths of time and then the culture died. Established cell lines that grew by attaching to the surface of the bottle or flask were uncommon. Efforts by many investigators had established that growing mammalian cells in culture was not the simple matter that characterized the laboratory growth of bacteria.

In a meticulous body of work, Eagle identified 27 components – 13 amino acids, 7 vitamins, glucose, and 6 salts – that in the presence of a small amount of human or bovine serum supported the *in vitro* growth of the murine fibroblast and human carcinoma cells. Omission of any one of the 27 components resulted in cytopathic effects that initially could be reversed by replenishing the missing component. If the missing component was not replaced, the culture died in two to three days. Eagle observed that the amino acid requirements were met only by the L-enantiomorphs but that the presence of the D-enantiomorphs were not inhibitory of the L-forms.

Eagle worked out the optimal concentrations of each of the 27 components and was struck by the similarity in the results for the murine and human cell lines. He inferred that all mammalian cells might have similar requirements for *in vitro* growth. This proved generally correct and led to Eagle's success in establishing many new lines of human and murine cells. Eagle's discovery of a chemically defined medium launched decades of extraordinarily productive investigations of mammalian cell metabolism, physiology and pathology, many carried out in his laboratory or by many of the scientists he trained. Eagle was the first to show that mammalian cells contain a pool of free amino acids and that the cells could maintain the pool against a concentration gradient. He also showed that a minimal intracellular concentration of each of the amino acids was required for protein synthesis and optimum cell growth. Eagle's lab carried out studies on the undefined components present in serum that were necessary for continued cell growth. Those studies launched the field of cellular growth factors. His laboratory made major contributions to understanding amino acid metabolism in mammalian cells and the effects of population density and contact inhibition on macromolecular synthesis.

Reference

Pernicious Anemia

Richard G. Lynch, MD
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MILESTONES


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In 1855, Thomas Addison at Guy’s Hospital described a lethal, idiopathic anemia that in 1872 was given the name pernicious anemia by Biemer. For decades following, a commonly held view was that pernicious anemia reflected the positive-acting, deleterious influence of an infectious agent or a microbial product. A popular concept was that the injurious agent caused excessive destruction of red blood cells.

Clinical and autopsy studies subsequently established that pernicious anemia was more than a disorder of red blood cells. Besides reduced numbers of blood erythrocytes and low concentrations of hemoglobin, patients were found to have excessive iron deposits in the liver, gastric glandular atrophy and achlorhydria, megaloblastic bone marrow hyperplasia, and profound demyelination and atrophy of sensory axons in the spinal cord. Under the microscope some cells of the body that normally turn over rapidly were found to be increased in size. Large erythrocytic precursors in the bone marrow were recognized early, but with time it became clear that the blood of patients with pernicious anemia contained giant granulocytes and platelets, and that large epithelial cells were present in their gastric and vaginal washings.

Beginning in the early 1900’s anecdotal reports suggested to a few investigators that pernicious anemia might be a nutritional deficiency disease. In the 1920’s two milestone discoveries were published, one by the pathologist George Whipple and colleagues at the University of Rochester, the other by two clinical investigators, George Minot and William Murphy, at Harvard Medical School. Whipple showed that anemia could be the result of a nutritional deficiency. Minot and Murphy developed a special diet that could reverse the pathology of pernicious anemia and cure patients. This was a tremendous breakthrough because 1-2% of adults over age 50, primarily people of northern European ancestry, suffered from this fatal disease. In 1934, Minot, Murphy and Whipple shared the Nobel Prize in Physiology and Medicine for their discoveries. Their Nobel lectures – available on the Nobel website (www.nobelprize.org) – provide interesting historical information and data about their experiments. These milestone discoveries launched decades of investigations, notably by W.B. Castle, that uncovered the complex pathophysiological mechanisms underlying pernicious anemia.

The importance of Whipple’s work was that it firmly established on a quantitative basis that the properties of food influenced blood formation, a concept not previously accepted. Whipple was originally interested in the metabolism of biliary and blood pigments and had developed a model of chronic anemia in dogs by repeated phlebotomy. When he began to investigate factors that influenced blood regeneration in chronically anemic dogs, Whipple focused on diet. Of the various diets tested, he found that liver and liver extracts were the most effective, although feeding other meats – kidney, muscle, or brain – also stimulated hematopoiesis. The choice of liver was fortunate. As others later pointed out, had Whipple fed iron salts to the dogs, he likely would have observed the same result since the dogs he studied undoubtedly suffered from iron deficiency anemia. Whipple’s findings on the effectiveness of liver feeding influenced Minot and Murphy to continue similar clinical studies they had been conducting in patients with pernicious anemia. A key element in the success of those studies was the reliance on blood reticulocyte counts to assess bone marrow responsiveness.

Once it was established that daily feedings of large amounts of liver or concentrated liver extracts induced varying degrees of remission in pernicious anemia, the central question became, “What is the active factor?” Castle discovered that daily administration by gastric tube of liquefied stomach contents from a healthy person removed an hour after ingestion...
of 300 grams of lean beef stimulated hematopoiesis in patients with pernicious anemia. Administration of gastric juice recovered from histamine-stimulated normal donors was ineffective. Administration of beef digested with pepsin was ineffective. Apparently, there was requirement for interaction between a factor in normal gastric juice and a factor in digested beef. The activity present in beef was designated as extrinsic factor; the activity present in normal gastric juice was designated as intrinsic factor. In retrospect, some of the dietary regimens in the clinical studies that led to the cure of pernicious anemia would probably raise eyebrows in today's Human Subject Committees.

Subsequent chemical analyses showed that extrinsic factor belonged to the cobalamin family of organometallic compounds. When it was shown that the active cobalamin was vitamin B12, therapy with vitamin B12 became standard treatment for pernicious anemia. Intramuscular treatment with vitamin B12 cured patients. To be effective by oral administration, vitamin B12 required the presence of normal gastric juice or massive doses of the vitamin. Later studies showed that intrinsic factor was a vitamin B12-binding protein produced by gastric gland parietal cells. Biochemical studies showed that vitamin B12 played a role in DNA synthesis, hinting at a mechanism that could account for the underproduction of red blood cell precursors in the bone marrow of patients with pernicious anemia.

Although curative treatment for pernicious anemia had been obtained, basic research in the area actually increased and publications continue through today. Uptake of vitamin B12 was shown to take place in the ileum via a specific mucosal receptor for the vitamin B12-intrinsic factor complex. New laboratory tests were developed to screen for pernicious anemia to distinguish it from other megaloblastic anemias. A great deal of effort was directed at understanding the basis for gastric gland atrophy and the loss of the intrinsic factor-producing parietal cells. The discovery of antibodies specific for parietal cells, intrinsic factor and other elements in the vitamin B12-uptake cascade have fostered the concept of pernicious anemia as an autoimmune disorder. Coming full circuit from the notion of a microbial etiology that was in vogue at the start of the 20th century and then discarded, it is now firmly established that Helicobacter pylori is a gastric pathogen that produces factors that are toxic for parietal cells.

Pernicious anemia is another example of a disease where an effective treatment came before an understanding of the underlying pathogenic mechanisms. It is another example where a disease, an experiment of nature, provided a powerful tool for biomedical discovery. Once the pathogenic mechanisms of pernicious anemia were understood, they provided critical insights into normal physiological processes, as well as the basis of other diseases. Minot, Murphy and Whipple worked without the highly specific, sensitive and sophisticated tools that are routine in today's biomedical research, yet they succeeded in making seminal discoveries, curing an incredibly complex disease, and launching the field of nutritional deficiency anemias.

References
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In their landmark publication, Kerr, Wyllie and Currie proposed that in addition to necrosis – cellular death that is a passive consequence of injurious agents such as ischemia, toxins, chemical and physical injury – a second form of cell death existed with features of an active, inherently controlled process. They termed this other form of cell death apoptosis, a Greek word used to describe the "dropping off" or "falling off" of petals from a flower.

It had long been assumed that cells must be lost continuously from many normal tissues to balance cell division and that loss of cells accompanies atrophy and physiological involution of tissues and organs. The terms "necrobiosis" and "shrinkage necrosis" were sometimes used to refer to this "physiological cell death" which was more a concept than a characterized process. Pathologists had recognized a non-inflammatory form of lymphocyte cell death in thymus glands undergoing stress involution and in reactive germinal centers of lymph nodes and spleens. Pyknotic nuclear fragments within germinal center macrophages – so-called "tingible body macrophages" – were readily detected by light microscopy, but their genesis and significance were unknown. Embryologists were familiar with a non-inflammatory form of cell death that occurred during morphogenesis, such as when lumens develop in the solid anlagen of ducts and intestine, and when inter-digital webs are resorbed during embryonic development of fingers and toes. Endocrine pathologists recognized a non-inflammatory form of cell death in adrenal cortical cells following withdrawal of ACTH.

The work that led to the concept of apoptosis came from the doctoral studies of the Australian pathologist John Kerr. Working with a rodent model of ischemic-induced hepatic atrophy developed decades before by the American pathologist Peyton Rous, Kerr observed individual, scattered hepatocytes that contained small, round cytoplasmic masses and fragments of pyknotic chromatin. These were located in the non-necrotic liver adjacent to zones of experimentally-induced ischemic necrosis. Much was already known about the morphologic features of necrosis because it typically involves large zones of dead tissue visible to the unaided eye, thus making it easy to obtain samples for analysis. In contrast, the single cell changes present in apoptosis are microscopic lesions that typically occur in one percent or less of the cells. In a series of electron microscopic studies published beginning in 1965 Kerr showed that the small, round, cytoplasmic masses present in individual cells consisted of membrane-bound cellular fragments containing crowded, but structurally well-preserved organelles and remnants of pyknotic chromatin. These findings were distinctly different from the autolytic, degenerative, vacuolar changes seen in electron micrographs of necrotic cells. From a morphologic perspective, necrosis is conspicuous; apoptosis is subtle.

In 1970 Alistair Currie, an endocrine pathologist and Professor of Pathology at the University of Aberdeen became aware of Kerr's work and invited him to spend a sabbatical in Scotland investigating the cellular changes that occurred during adrenal cortical atrophy. Kerr joined Currie, and along with Andrew Wyllie, a Ph.D. student in Currie's lab, they found the same ultrastructural changes in adrenal atrophy that Kerr had described in the atrophic liver. Allison Crawford, a developmental biologist and Ph.D. student in the Aberdeen pathology department at that time, drew the group's attention to the extensive literature on "programmed cell death" that occurs during mammalian embryogenesis. Few outside of the field of developmental biology were aware of that literature and the published electron micrographs showing cellular changes during embryonic organogenesis that were the same as Kerr had observed in his liver model.

The application of electron microscopy by Kerr, Wyllie and Currie provided the resolving power to visualize the cellular changes that distinguished the two forms of cell death.
Apoptosis in normal tissues characteristically affects scattered single cells, thus limiting the opportunity for detection and investigation by microscopy. Certain physiological and pathological conditions increase apoptosis and these provided the models studied by Wyllie, Kerr and Currie. In a series of publications they described the evolution of apoptosis in normal neonatal rat adrenal cortex, in embryonic mesenchyme, in both human and animal neoplasms, in the adrenal cortex following ACTH withdrawal, and in various types of liver and adrenal injury. In every case the ultrastructural features were essentially the same. The authors designated the small, roughly spherical or ovoid cytoplasmic fragments as apoptotic bodies. The electron microscopy studies showed that the structural changes in apoptosis take place in two distinct stages: the first comprises the formation of apoptotic bodies, the second their phagocytosis and degradation by other cells, sometimes macrophages, sometimes parenchymal cells, sometimes both.

Subsequent studies by Kerr provided a possible explanation for the high mitotic rate, but paradoxically slow growth of certain malignant tumors, such as human basal cell carcinoma. As a surgical pathology fellow I was taught to ignore mitotic figures in assessing malignant tumors because they were unreliable predictors of tumor growth. At the time that was sound advice because trying to predict the growth properties of a tumor knowing its mitotic index but not its apoptotic index was like trying to predict the overall rate of a chemical reaction knowing only the forward rate, but not the backward rate.

The importance of the publication by Kerr, Wyllie and Currie was that it presented an integrating concept that fundamentally changed thinking about cellular kinetics. The authors proposed – and provided supporting evidence – that cell death by apoptosis was a normal, intrinsically controlled, active process that occurred during physiological and pathological conditions and played a complementary but opposite role to mitosis in the regulation of animal cell populations. It is amazing that their ground-breaking publication was essentially ignored for more than a decade. Then, in the late 1980’s publications from numerous laboratories began to describe some of the biochemical and molecular genetic mechanisms involved in apoptosis. During the 1990’s the field underwent an explosive rate of growth and it was difficult to pick up a biology journal that did not have at least one article on apoptosis.

Many of the historic details of the research by Kerr, Wyllie and Currie that launched the current era of apoptosis research and led to the molecular genetic studies that followed in laboratories around the world are nicely covered in the article by Cummings, Winterford and Walker. The 1972 publication by Kerr, Wyllie and Currie is a prototype of a paradigm-changing article. The history of the discovery of apoptosis and the central role played by trainees working with seasoned mentors should be inspirational for graduate students and beginning investigators.

References