Introduction

*Milestones in Investigative Pathology* is a collection of articles written for the American Society for Investigative Pathology (ASIP) newsletters over the past 15 years. Originally the brainchild of the late Dr. Richard G. Lynch, and currently written by William K. Funkhouser, MD, PhD, these *Milestones* articles briefly summarize seminal research findings spanning the 17th to 20th centuries 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 articles in this second volume - *Milestones in Investigative Pathology (2010 - 2015)* - appeared in *ASIP Pathways* from 2010 - 2015, and were written by William K. Funkhouser, MD, PhD. Both volumes are available as PDFs for download from the ASIP website at www.asip.org/publications/milestones.

It is our hope that the *Milestones* compendia will continue to 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
# Table of Contents

Introduction ..................................................................................................................................................................i

Edward Jenner and Vaccination: The Road to Elimination of Epidemic Smallpox  
(ASIP Pathways Vol. 5 No. 1 February 2010).............................................................................................................1

Asbestos and Mesothelioma  
(ASIP Pathways Vol. 5 No. 3 September 2010)..........................................................................................................5

The Role of the Thymus in Lymphocyte Development  
(ASIP Pathways Vol. 6 No. 2 June 2011) .................................................................................................................7

Semmelweis and Puerperal Fever  
(ASIP Pathways Vol. 7 No. 1 February 2012).............................................................................................................9

Proving Virchow's "Omnis cellula e cellula" ("All cells derive from other cells")  
(ASIP Pathways Vol. 7 No. 2 October 2012)................................................................................................................11

About the Pathologic Coagulation Functions  
(ASIP Pathways Vol. 8 No. 2 August 2013)..............................................................................................................13

Microscopes, Bacteria, and DNA: Connecting Three Centuries of Scientific Discovery  
From van Leeuwenhoek and Hooke to Flemming and Avery  
(ASIP Pathways Vol. 9 No. 1 February 2014)................................................................................................................15

Whistleblowers of the 1950s: Epidemiologists and the Association Between Cigarette Smoking and Increased Risk of Lung Carcinoma  
(ASIP Pathways Vol. 10 No. 1 January 2015)................................................................................................................17
Edward Jenner and Vaccination: The Road to Elimination of Epidemic Smallpox

William K. Funkhouser, MD, PhD

Originally published in ASIP Pathways, Volume 5, Issue 1 - February 2010

MILESTONES

1. Jenner E: An Inquiry into the Causes and Effects of the Variolae Vaccinae, A disease discovered in some of the western counties of England, particularly Gloucestershire, and known by the name of England, particularly Gloucestershire, and known by the name of the Cow Pox. In:London: Sampson Low, 1798

When we stand in line waiting for our H1N1 influenza vaccination, and when we have our children vaccinated against previously common childhood infections, we act on the belief that prophylactic immunizations will prevent specific infectious diseases. We make assumptions, e.g., that microbiologists have purified the etiologic agent, that immunologists have identified dominant immunogenic epitopes for B and T lymphocytes, and that clinicians can determine appropriate age, routes, doses, and booster dose intervals to maximize population resistance to the spread of these diseases. We assume that the risk of immunization complications is low compared to the risk of morbidity and mortality associated with the diseases we want to prevent. The net result is that routine prophylactic mass immunization programs have reduced incidence of many of these infections into the realm of rare, call-the-ID-Fellow diseases. This is great news, and gives us a chance to live long enough to procreate and then die of something else. But how did we get to this era in history, wherein epidemics of infectious diseases can be prevented?

Smallpox infection has plagued man for at least 3 millenia. Evidence of probable smallpox infection was found in the mummified Egyptian pharaoh Ramses V from 1157 BC (www.microbiologybytes.com/virology/Poxviruses.html). Recognition of the morbidity and mortality of smallpox in Asia and the MedEast led to empirical prophylactic immunization with smallpox (variola) ulcer debris (‘variolation’) via respiratory, gastrointestinal (GI), and skin portals by the first millennium AD. The limited appeal of variolation in Western Europe and England was boosted in 1722 when children in the English royal family were variolated. Voltaire wrote in 1732 (www.bartleby.com/34/2/11.html) that smallpox outbreaks infected an estimated 60% of unvariolated people, and killed a third of those infected. As prophylaxis, variolation reduced smallpox mortality from 20% to around 2%. However, it was neither risk-free nor 100% effective, leading to post-variolation smallpox in some patients and to ineffective prevention of subsequent smallpox in others. Thus, the stage was set for development of a less risky, more effective alternative to variolation.

Dr. Edward Jenner was a rural physician who had been trained to be an experimentalist by Dr. John Hunter. Jenner recalled being variolated as a child, and claimed that it almost killed him. His clinical practice proximity to dairy farmers allowed him to hear firsthand the folklore that farmers and milkmaids exposed to cowpox had a decreased risk of developing smallpox. Just as Dvorak crafted folksongs into the New World Symphony, Jenner crafted folklore into a testable hypothesis about prevention of smallpox through vaccination with cowpox.

In his 1798 paper (www.bartleby.com/38/4/1.html), he discussed cowpox and smallpox viruses as somehow related, and described the results of a 1796 uncontrolled vaccination trial on children using ulcer debris from cowpox patients. He observed that these children had limited responses to subsequent exposure to smallpox, and interpreted this as a protective effect due to the prior cowpox vaccination. Subsequent larger trials by others obtained confounding results, possibly due to vaccine contamination with smallpox, or to use of skin ulcers due to other organisms. However, preventive vaccination against smallpox was so attractive that mass vaccination programs, e.g., of the Napoleonic army, occurred within the next 10 years -- without a marketing budget.

Dr. Jenner benefited from training that emphasized hypothesis-testing through
experimentation. He observed the presence of a clinically similar disease in his clinical practice environment. He noted the current acceptance of variolation in medical practice, and sought to improve the method. The lessons to be learned from Jenner’s work are 1) that chance favors a prepared mind (Jenner knew about variolation inoculation techniques); 2) that one should be open to new ideas, however iconoclastic, based on direct observation (Jenner confirmed the folklore about milkmaid’s resistance to smallpox); 3) that great benefit is perceived with an effective new approach to a terrible disease (smallpox was so feared that Jenner vaccinated his own son as part of the initial cohort).

During the early 19th century, vaccination against smallpox evolved into direct vaccination, arm-to-arm, from individuals with presumed cowpox infection, without interval purification. The original immunogen was cowpox, but cowpox mimics exist in cattle, and definite cowpox infection was an uncommon infection. Vaccines coming to the United States by the mid-19th century are Orthopoxvirus ([www.ictvonline.org/virusTaxonomy.asp?version =2009&bhcp=1](http://www.ictvonline.org/virusTaxonomy.asp?version=2009&bhcp=1)). It is unknown whether vaccinia virus evolved from cowpox, pox viruses in horses, or as an attenuated variant of smallpox itself. What is known is that smallpox is a 186 kb double stranded DNA poxvirus that contains 187 major open reading frames, and that it generates an active, non-latent infection limited to humans. Forty-nine of the smallpox proteins are now known that the most effective live virus vaccines were those at the protein level suggest that vaccinia and camelpox viruses are the most phylogenetically similar to smallpox. (In case you’re wondering, chickenpox (varicella-zoster) is a herpes virus, not a poxvirus. It’s part of how Infectious Disease keeps the rest of us from other herpes viruses.) Vaccinia is closer in size (190kb) to smallpox than is cowpox (224kb). Phylogenetic studies at the protein level suggest that vaccinia and camelpox viruses are the poxviruses most closely related to smallpox. So 19th century smallpox vaccines were empirically selected for effectiveness, and we now know that the most effective live virus vaccines were those most phylogenetically similar to smallpox. (In case you’re wondering, chickenpox (varicella-zoster) is a herpes virus, not a poxvirus. It’s part of how Infectious Disease keeps the rest of us confused.)

Extension of the concept of disease prevention by vaccination blossomed during the last half of the 19th century, along with cell theory and infectious agent purification and description. In 1880, Dr. Louis Pasteur published his work on the use of ‘attenuated’ cultures of the diplococcus causing chicken cholera ([Pasteurella multocida](http://www.ictvonline.org/virusTaxonomy.asp?version=2009&bhcp=1)) to prophylactically immunize chickens against lethal infection. Pasteur recognized the similarities to Jenner’s work 80 years prior, but improved the method by performing controlled experiments on an animal model using a purified immunogen.

To summarize, Jenner had used the clinically similar cowpox ulcer debris to generate cross-reactive immunity to smallpox virus. Subsequent use of vaccinia virus for mass vaccination programs settled on a poxvirus even more similar to smallpox than cowpox. Pasteur introduced use of purified, attenuated bacteria to generate specific immunity to chicken cholera. Each of these approaches capitalized on organisms that could infect, but not kill, the host. Thus, early experiments showed that an ideal vaccine maximizes immunogenicity and minimizes pathogenicity. What neither scientist understood was the mechanism of specific immunity, and how vaccination prophylactically amplified cellular and/or humoral responses to specific organisms.

Experimental studies to understand intra- and inter- species tissue transplantation led to definition of the barcode for individual identity, the gene products of the major histocompatibility complex (H-2, MHC, HLA) locus. Dr. Georg Schöne outlined laws of transplantation in 1912, i.e. that xenografts invariably fail, allografts usually fail, and syngeneic grafts usually succeed. Drs. Little and Snell of the Jackson Lab hypothesized that there were multiple histocompatibility loci, each codominantly expressed, and that expression of these MHC gene products by the donor, but not by the recipient, would lead to rejection. They generated congenic mouse strains, differing only by individual major or minor histocompatibility loci, and demonstrated that MHC differences defined the probability of graft rejection.

Dr. Peter Medawar used a rabbit skin transplant model in the early 1940s to demonstrate that the skin graft rejection inflammatory infiltrate is comprised of lymphocytes and macrophages, and that a subsequent second skin graft from the same donor was rejected more rapidly than the first. (The critical role of macrophages as antigen-presenting cells will be a topic for a future Milestones article on Matchinkoff and phagocytes.) Thus, the host immune response to an allograft has a memory for the allo-MHC proteins.

Zinkernagel and Doherty showed in 1974 that a similar T-cell mediated killing process occurs during recognition of viral infected cells. Using lymphocytic choriomeningitis (LCM) virus as a model infectious agent in mice, they demonstrated that lymphocyte killing was restricted by recognition of their own MHC molecules on macrophages and other virally infected cells. T lymphocytes therefore activated only to self MHC + viral antigen. Medawar’s T lymphocyte response to allo-MHC in rabbit skin grafts could then be viewed as a version of self MHC + antigen. Subsequent work showed that the antigenic proteins are not exhibited whole, but rather must be cut up (processed) and bound as short polypeptides to self MHC class I and class II molecules (presented) on the cell surface (reviewed in 11,12). The model is that MHC class I+ cells (antigen presenting cells) first activate viral epitope-specific ‘helper’ (CD4+) T lymphocytes, which secrete lymphokines that in turn allow activation and proliferation of viral epitope-specific ‘cytotoxic’ (CD8+) T lymphocytes and viral epitope-specific B lymphocytes. Infected MHC class I+ cells are then recognized by these viral epitope-specific cytotoxic T lymphocytes, and free virus can be bound and cleared by viral epitope-specific immunoglobulin. Thus, transplantation studies led to an understanding of the barriers posed by MHC, and that allo- and xeno- transplantation barriers are perceived by the specific immune response in the same way as xeno-infectious agents, i.e. as non-self.

A subset of the T lymphocytes activated in response to the viral antigens is long-lived, and retains its specificity for the cognate antigen. For the anti-vaccinia response, circulating CD4+ T lymphocytes can be activated correctly over 35 years after childhood immunization against smallpox, with one limiting dilution estimate of 3 CD4+ T cells per 10⁶ CD4+ lymphocytes. Like the allograft response, the host response to virus has a memory, and it is these memory cells that allow the individual to mount a more rapid secondary response to the infectious agent.

In theory, a robust vaccine would be processed and presented by all MHC class I and II haplotypes, would trigger memory T- and B- lymphocyte formation, and would not require booster immunization. In practice, insufficient processing/presentation,
insufficient T cell numbers, or insufficient T cell function (HIV, iatrogenic immunosuppression) could each lead to an inability to respond to antigen and generate memory of the antigen. This could cripple the host's ability to respond to the infectious agent, including responding to attenuated live organism vaccines. In fact, some children vaccinated with vaccinia virus developed progress vaccinia, occasionally with bad complications\(^4\). This would be expected, based on the intricate requirement for protein processing and polypeptide presentation on MHC molecules to T lymphocytes. Fortunately, most individuals can process and present whole-organism vaccine proteins, and can amplify a virus-specific immune response and memory cell formation before the actual pathogen is contacted.

If live organism vaccines pose a real but unpredictable risk of clinical infection and death, then perhaps vaccine engineers can identify polypeptides that are produced in the organism, and which would act as dominant epitopes for T- and B- lymphocyte activation. Individuals able to present these polypeptide epitopes sufficient to trigger T cell activation should be protected from subsequent infection by earlier, more intense secondary response via memory lymphocytes and circulating antibody. The challenge will be to identify sets of dominant epitopes that can be presented by all HLA haplotypes in the outbred human population.

Whether whole-live, whole-killed, or as dominant polypeptides, the timing of the secondary response is critical. For smallpox, new exposure starts the clock ticking, with the 7-10 days required to mount a primary specific immune response too close for comfort to the 12 day incubation period before the first viremia. When this race occurs between a non-immune individual and the smallpox virus, 30% of infected individuals die. The more immediate secondary response, and the presence of circulating anti-viral antibody, allow more of the vaccinated population to survive. Therefore, prophylactic immunization prepares the individual’s specific immune system to respond promptly and decisively to subsequent infection.

Can mass vaccination reduce disease incidence to zero? The World Health Organization (WHO) decided to eradicate smallpox in 1958. WHO-sponsored vaccinia vaccination programs against smallpox in the 1960s eradicated clinical smallpox from the earth, with the last reported case of non-lab-acquired smallpox in October 1977. As of 1980, smallpox existed only in research labs in the US and the USSR. (We can only hope that these stocks are secure, because none of our children have been vaccinated against smallpox.) And nature continues to test our ability to keep up with viral mutation rates and manufacture specific vaccines, as evidenced by the annual challenge of predicting the Hexoseaminidase-Neuraminidase combination for influenza vaccines. New viruses also adapt to humans, e.g. HIV, which began to be clinically significant just as clinical smallpox was extinguished.

Dr. Jenner was, and we are, fortunate that smallpox virus had no non-human reservoir, had no asymptomatic human carrier state, and that it had a close poxvirus relative (cowpox) with a local occupational association, similar skin signs of infection, and viral epitopes that led to a cross-reactive specific immune response to smallpox. Serendipity, indeed! Pasteur improved the method through use of attenuated variants of the purified agent itself. Jackson Labs’ inbred mouse strains led us to understand class I and class II MHC expression, i.e., the molecular basis of self. Medawar showed that the immune response to allograft has memory that speeds the secondary exposure to antigen. Doherty and Zinkernagel demonstrated the MHC-restricted nature of T cell activation to macrophages and other virally infected cells, and Demkowicz documented CD4 T lymphocytes memory lasting decades after childhood vaccination with vaccinia. Convincing bench, clinical, and epidemiologic data, along with international commitment, allowed elimination of epidemic smallpox on our planet in our lifetime. We did it, and we thank you, Dr. Jenner.

P.S: If you're wondering what the Latin quote on the cover page of Dr. Jenner's 1798 paper ("Quid nobis certius ipsis sensibus esse potest, quo vera ac falsa notemus") means, it translates as "What, more certain than our senses themselves, can there be, by which we indicate truths and falsehoods?"

Web-based resources for additional reading:
www.nlm.nih.gov/exhibition/smallpox/
www.microbiologybytes.com/virology/Poxviruses.html
www.bt.cdc.gov/agent/smallpox/vaccination/live-virus.asp

References:

Milestones in Investigative Pathology, 2010 - 2015, William K. Funkhouser, MD, PhD
Copyright 2016, American Society for Investigative Pathology

3
Asbestos and Mesothelioma

William K. Funkhouser, MD, PhD
Originally published in ASIP Pathways, Volume 5, Issue 3 - September 2010

MILESTONES


As you endure another ad extolling the virtues of legal counsel for mesothelioma patients, you could assume that we’ve known since the Industrial Revolution that asbestos causes mesothelioma. Au contraire. We didn’t know that asbestos pneumoconiosis was associated with increased risk of mesothelioma until Dr. Wagner suggested it in 1960. The above article, available at http://imig.org/wp-content/uploads/2010/03/Wagner_Historic-Meso-Article_1960.pdf, is the first in a series of critical contributions that demonstrated the causal association of asbestos fibers and mesothelioma.

Pathologists of the 1930s and 40s were sorting out the biphasic morphologic potential of anecdotal cases of mesothelioma (reviewed in 2). In these early case reports, etiology was not postulated, occupational history was not recorded, and ferruginous bodies were not described.

Industry was well aware of the insulation and fire-retardant qualities of asbestos, and used it extensively in construction and ship-building in WWII. One patient in our hospital remembered shipyard construction duty where “there was so much asbestos around that we had snowball fights with it.”

J.C. Wagner was a pathologist working in South Africa from 1951-62. The story goes that the initial observation is traced to an autopsy on an asbestos miner in 1956, in which he identified asbestos fibers in a gelatinous pleural neoplasm (http://imig.org/about/wagner-award-recipients-2/j-christopher-wagner-biography). He sought out other cases of mesotheliomas, and reported a cluster of patients with mesothelioma who had been physically close to asbestos mining or milling. In this initial paper, he gives a history of asbestos mining and milling in South Africa, indicating a transition from manual separation of fibers (“cobbing”) to automated milling in around 1915. 32 of the 33 patients he presented were either miners, millers, or children exposed to dusts from these industries 20-40 years prior. His initial paper 1 was observational, and hypothesized a higher-than-expected probability of mesotheliomas in individuals exposed to asbestos.

Dr. Wagner recognized the need to prove an etiologic relationship between asbestos and mesothelioma. He published data in 1962 on rats inoculated in the pleural space with suspensions of different types of asbestos. 3 of 50 (6%) rats receiving crocidolite (“blue” asbestos) or chrysotile (“white” asbestos) developed pleural mesotheliomas, said to show similar morphologies to human mesotheliomas. After moving to the UK in 1962, he published data in 1969 on rats inoculated in the pleural space with 20 mg of crocidolite, chrysotile, or amosite fiber suspension, then followed to natural death. 30-40% of amosite-exposed rats, and 50-70% of the crocidolite- and chrysotile- exposed rats, developed pleural mesotheliomata. None of the saline inoculated controls rats developed mesotheliomas. Rats with mesotheliomas died at 500-750 days of age, whereas control rats died at around 1,000 days. (No statistical testing was performed on the datasets in either the Nature or Br J Ca papers, so take heart, you qualitative types out there.)

Dr. Wagner recognized that pleural inoculation experiments were unrealistic, so he followed up his pleural inoculation experiments with dust aspiration experiments. Exposures mimicked 7 hour/5 day per week work hours. Amosite was the least fibrogenic of the three. As expected, increasing exposure led to increasing fiber load in the lungs. Interestingly, there were marked differences in steady-state fiber load following inhalation of similar amounts of crocidolite and chrysotile dust, indicating difference in dust clearance rates. Although chrysotile was cleared much better than crocidolite, the incidence of mesothelioma (6%) was similar to that seen with high-fiber...
burden crocidolite (6%). Most mesotheliomas developed after 6 months of dust exposure but, astoundingly, 2 rats exposed for only one day each (one to amosite and one to crocidolite) developed mesotheliomas. Assuming careful control of experimental conditions, these data beg the question of whether there is any risk-free exposure to asbestos dust.

Formal handling of asbestos exposure data in humans was sought by the NIH for a case-control study of mesothelioma patients presenting between 1975 and 1980 from three different populations (LA, NY, and the VA system). Dr. Wagner served as the study Pathologist. Their data showed that "90% of the incidences of pleural mesothelioma among men were directly attributable to past exposures to asbestos." They found an odds ratio of 27-fold for mesotheliomas in individuals with prior asbestos exposure. Statistical analysis confirmed what Dr. Wagner had suspected with that first autopsy.

In summary, Dr. Wagner made the initial association of occupational or environmental exposure to asbestos with subsequent risk for development of mesothelioma. This association required a prepared scientific mind in a region where there was high exposure to the etiologic agent. Following Koch's postulates, he showed that exposure of an animal model to the purified putative etiologic agent increased the incidence of a disease that was rare in the untreated control animals. His experimental work was confirmed by the exposure of humans involved in industries involving insulation and fire retardant materials. Careful history-taking was critical to making the observation, as the delay from exposure to signs/symptoms is measured in decades.

The scientific demonstration of asbestos as the major etiologic agent in mesothelioma prompted marketing of legal recourse to patients receiving this diagnosis. Asbestos has no medicinal value to the human, so our legal system recognized a skewed risk:benefit ratio to asbestos exposure, and proceeded to bankrupt the asbestos industry. Isn't this interesting, that we've witnessed destruction of a legitimate insulation/fire retardant materials industry because its dusty product leads to around 90% of the cases of a rare disease, when we've knowingly tolerated tax revenue subsidy of the growth, processing, and marketing of tobacco, whose dusty product leads to around 90% of the cases of the tobacco-associated common diseases, chronic obstructive pulmonary disease (COPD) and primary lung carcinoma?

References:
The Role of the Thymus in Lymphocyte Development

William K. Funkhouser, MD, PhD
Originally published in ASIP Pathways, Volume 6, Issue 2 - June 2011

MILESTONES

1. Miller JFAP: Immunological Function of the Thymus.
   Lancet 1961, 278:748-749

   Ann NY Acad Sci 1962, 99:340-354

   Nature 1967, 216:659-663

As you recover from your latest workplace cold virus infection, you should thank Dr. Jacques FAP Miller for discovering the role of the thymus in lymphocyte development.1,2,3

Now you can understand how the viral infection was cleared, even if you can't speed the process. Before 1961, the thymus was thought to be irrelevant to the cellular immune response, likely because of the absence of secondary lymphoid follicles, the absence of measurable effects after adult thymectomy, and the general absence of immunologic reactivity of thymocytes4. Circulating small lymphocytes were thought to be a homogeneous population able to generate both cellular and humoral immune responses. Dr. Miller's discoveries clarified the role of thymus-derived (T) lymphocytes in generation of an initial immune response to antigen, and the dependence on T lymphocyte function for antibody production. His work has led to our view of a unified role for T lymphocytes in the specific cellular immune response to non-self, whether virus or allograft.

Dr. Miller was an intentional scientist, but an unintentional immunologist, discovering thymus function while trying to study leukemogenic viruses in mice1. One arm of one experiment required thymectomy on neonatal mice, a novel surgical procedure that he was the first to perfect. His early observation was: "Between 50 and 120 days of age, about 70% of the mice in the neonatally thymectomized group developed a syndrome characterized by progressive wasting, lethargy, ruffled fur, hunched posture, diarrhea, and death within 1-3 weeks. This syndrome was rare in mice thymectomized between 1 and 3 weeks of age, and has never been seen in mice thymectomized after 3 weeks of age"5. The wasting was preceded by decreased lymphocyte concentration in peripheral blood, and absence of secondary follicle formation in secondary lymphoid organs5. He noted that this wasting syndrome was less common if the neonatally thymectomized mice were raised in cleaner conditions. He concluded that "mice without a thymus from birth were susceptible to infection." We could add that the importance of the thymus for generating xenoreactive lymphocytes decreases over time after birth.

It was known by the mid-1940s that skin graft rejection had a predictable time course, was a function of Major Histocompatibility Complex (MHC) differences, and was lymphocyte-mediated6. However, it was not known in 1961 that the thymus had a role in allograft rejection. Dr. Miller did skin graft experiments from other strains of mice and rats onto mice that had been thymectomized at different times. He found that neonatally thymectomized mice tolerated both allo- and xeno- skin grafts (most grafts survived >50 days), whereas thymectomy after 3 weeks of age showed normal allograft rejection (most grafts survived <25 days)7. Different colors of donor fur illustrated the tolerance of neonatally-thymectomized mice for allo- and xeno- skin grafts. As an example of following through on Koch's postulates, he injected splenocytes pre-sensitized to one of the skin grafts into a stable adult skin graft recipient, and saw rejection of the allograft within 12 days. He concluded that the neonatal thymus plays a critical role in transplantation rejection, noting that "thymectomy of the neonatal mouse is associated with marked depletion in the lymphocyte population and serious impairment of the maturation of the faculty for transplantation immunity"8.

Dr. Miller proceeded to show by 1967 that there are two major subsets of lymphocytes with different functions. His experiments dissected the role of thymocytes and bone marrow cells in the generation of antibody. Using a model of immune response to sheep red blood cells (RBC) in mice, he found that sensitization of thymocytes to sheep RBC was required, and that sensitized splenocytes from these sRBC-immune mice required bone marrow cells to generate hemolysin (antibody). Neither thymocytes nor bone marrow cells alone were
pluripotent, and both together were required in this model to
generate antibody. He concluded that "sRBC antigen-reactive
cells are the progeny of antigen-independent precursor cells, the
differentiation of which is dependent on the thymus. Our failure to
detect precursors of antigen-reactive cells in populations of
thymus lymphocytes would suggest that the precursors of the
hemolysin-producing cells are derived from marrow. In this one
set of elegant experiments, Dr. Miller dissected out the existence
and roles of the two major types of lymphocytes, "T" for thymic
and "B" for bursal or bone marrow. His work laid the groundwork
for others to dissect the cellular interactions in the thymus that
facilitate self-tolerance, the molecular events leading to lg and T-
cell receptor rearrangements in the primary lymphoid organs, the
protein level process of mature T cell activation upon binding the
2-body ligand of self-MHC and processed antigen, the role of
soluble lymphokine in mature T- and B- lymphocyte activation, and
the longevity of memory T cells associated with thymic involution
by adulthood.

So, take your pseudoephedrine for your cold, and head back to
work. But instead of thanking your pharmacist, please thank Dr.
Jacques Miller, who has helped you to understand the major
lymphocyte subsets that drive your specific immune response to
rhinoviruses, and your T cells, which allow you to control the
infection itself.

References:
1. Miller JFAP: Immunological Function of the Thymus. Lancet 1961,
   278:748-749
   Ann NY Acad Sci 1962, 99:340-354
3. Miller JFAP and Mitchell GF: The Thymus and the Precursors
4. Miller JFAP: Discovering the Origins of Immunological
5. Medawar PB: A Second Study of the Behavior and Fate of Skin
   Homografts in Rabbits: A Report to the War Wounds Committee
While you read the published results of clinical trials, and while you discuss experimental design and data sample comparisons with your biostatistician, you should tip your hat to Dr. Ignaz Semmelweis, who crafted and documented an unintentional 1847 clinical trial that defined and prevention of puerperal fever of women and their newborns.

As an obstetrician in Vienna in the mid-19th century, Dr. Semmelweis was confronted by annual puerperal fever mortality rates of up to 16%. Current thought leaders speculated that the etiology of puerperal fever was different for different patients, and that it was some combination of climate, ventilation, diet, overcrowding, patient fear/injured modesty/social status, or national differences in quality of obstetrics practice. If this multivariable explanation failed to convince the uninitiated, thought leaders assigned the etiology to “atmospheric – cosmic – telluric (terrestrial) influences,” which would seem to trump all other possibilities.

After graduating from medical school at the University of Vienna in 1844, Dr. Semmelweis trained as an obstetrician at the Vienna General Hospital. By government edict in 1840, the obstetrical service consisted of two Divisions, differing only by who staffed the clinics and wards. The 1st Division was staffed by medical students, and the 2nd Division was staffed by midwife students. The services were otherwise identical with respect to physical plant, temperature control, demographics/social status of the women admitted to the service, bed density, quality of obstetrical care and procedures, hygiene of bed linens, patient diets, etc. This created a clinical experiment that controlled for all variables except staff and staff duties. Semmelweis made a series of critical observations. He noted that puerperal fever was associated with prolonged (>24h) dilation phase of labor of women on the 1st Division service, but not on the 2nd Division service. He noted that death from puerperal fever was more common in both women and their newborns on the 1st Division service. He noted that autopsy findings in affected women and their affected newborns were similar, arguing for a common etiology. He noted that puerperal fever was sporadic rather than generalized in crowded wards, arguing against patient-to-patient contagious transmission. He noted that puerperal fever incidence was independent of season, bed location, ventilation, room temperature, diet, frequency of religious rites, and patient social status. Although he did not document the data for comparison, he noted that women who delivered in transit to the hospital (“street births”) had lower rates of puerperal fever than women attended to by medical students in the 1st Division. He formalized his observations by documenting annual mortality rates for the two Divisions during the period 1841-46, showing that 1st Division (staffed by medical students) had an average mortality rate due to puerperal fever of 9.9%, while the 2nd Division (staffed by midwife students) had an average mortality rate due to puerperal fever of 3.4% (see Table 1). Semmelweis recognized that the only difference between the two Divisions was the staff, and he recognized that the only difference in staff duties was the involvement by medical students in autopsy examination. He noted that (gentlemen) medical students saw no need to wash their hands between patients. His hypothesis was that puerperal fever was due to “conveyance of decomposed animal-organic matter from without,” meaning from autopsy patient via medical student to obstetric patient.

Semmelweis should have thanked the Hapsburg Empire for their imperial edict of 1840 that stratified staffing of the 1st and 2nd Obstetrics Divisions. From 1833 to 1840, these two Obstetrical Divisions had been staffed by a mixture of medical students and midwife students. This allowed Semmelweis...
to compare his 1841-46 puerperal fever mortality data to earlier 1833-40 puerperal fever mortality data. He noted that puerperal fever mortality rates 1833-40 (during a time when each Division was staffed by both medical students and midwife students) were comparable (average 6.6% in 1st Division, and average 5.6% in 2nd Division). He concluded from these sample comparisons that puerperal fever was due to “conveyance (by medical students) of decomposed animal-organic matter from without.” The only improvement in trial design that we could offer 160 years later would be to document and compare puerperal fever mortality rates in the control group of women who delivered before reaching the hospital. Semmelweis practiced before the isolation of bacteria (van Leeuwenhoek’s ‘animalculae’ from ca. 1673) and the evolution of germ theory, so it was left to Koch in ca. 1870 to isolate the particular streptococcus in women with puerperal fever, and to Pasteur in 1879 to culture the particular streptococcus from the blood of women with puerperal fever.

Semmelweis anticipated Koch’s postulates (1890) by 40 years, in the sense that his presumed etiologic agent could be proven if puerperal fever mortality dropped following elimination of the putative etiologic agent. Semmelweis did this experiment starting in 1847 by interrupting the transmission of the “decomposed animal-organic matter” by requiring handwashing in calcium hypochlorite (chlorine bleach) between cases. He collected and documented puerperal fever mortality data for the period 1847-58 (Table 1). He found that puerperal fever mortality rates in the 1st Division fell to an average of 3.6%, comparable to the 3.1% seen in the 2nd Division. He concluded that puerperal fever mortality could be reduced by interrupting the “conveyance of this decomposed animal-based organic matter” from autopsy suite via medical student to obstetrics ward. We don’t know why he chose calcium hypochlorite as a proto-antiseptic, although he commented that “it eliminated the smell on the hands.” Here’s to the archencephalon as a trigger for empirical science.

Semmelweis published his initial observations in a Viennese medical society journal (Zeitschrift d. k. k. Gesellschaft d. Ärzte in Wein 4, pt 2: 242, 1847-48), but his conclusions were not widely disseminated, not understood, not confirmed, and/or not embraced, because practice habits did not change. He published ‘open letters’ to other obstetricians to promote his perspective, but practice habits did not change. He died in an asylum in 1865 (his wife had him committed, so be careful out there), possibly following inoculation at his last autopsy. His erstwhile reputation was rebuilt only after Koch’s isolation of streptococcus from a woman with puerperal fever ca. 1870 and Pasteur’s culture of streptococcus from the blood of a woman with puerperal fever in 1879.

This history of Semmelweis and puerperal fever contains several practical teaching points. Semmelweis capitalized on an opportunity to gather data in a government-regulated practice environment that controlled for all variables except staff and their duties. He measured and trended mortality data to show by sample comparison that increased risk for puerperal fever was correlated with manual examinations of peripartum women by medical students with contaminated hands. He postulated a single etiology for all peripartum women and their newborns, postulated the mechanism of exposure to this etiologic agent, and interrupted this process of manual inoculation of peripartum women by use of a proto-antiseptic hand wash. Although not accepted at the time, his work presaged the work of Koch, Pasteur, and Lister with regards to microbial infection, prophylaxis, and Koch’s postulates for defining etiologic micro-organisms. Semmelweis’s persistent defense of his data-driven conclusions, in the face of an unaccepting medical community, is laudable. However, like the rest of us, Semmelweis was not perfect. Although a careful student of data, he didn’t like to write, delaying by 14 years the full publication of his critical work. Had he published in the peer-reviewed, widely-read literature of his era, antiseptic approaches would probably have been adopted well before Lister, saving the lives of thousands of peripartum women. Semmelweis’s letters to practicing obstetricians, full of ad hominem arguments and thinly veiled accusations of murder, did not strengthen his argument. Finally, his book would have benefited from careful editing – it runs for several hundred pages, is frequently redundant, and could have been collapsed into a five-page paper in a modern journal. Ignore your editor at your peril.

| Table 1: Mortality from Puerperal Fever (Mean Range) |
|---|---|
| Vienna General Hospital |
| | 1st Division | 2nd Division |
| 1833-40 | 6.56% (3.04, 9.09) | 5.58% (2.26, 8.60) |
| 1841-46 | 9.92% (7.80, 15.75) | 3.38% (2.03, 7.59) |
| 1847-58 | 3.57% (1.27, 9.10) | 3.06% (1.33, 6.18) |

References:
Proving Virchow’s “Omnis cellula e cellula” (“All cells derive from other cells”)

William K. Funkhouser, MD, PhD
Originally published in ASIP Pathways, Volume 7, Issue 2 - October 2012

As you count mitotic figures per 10 high-power fields, your activity presumes knowledge that the gross tumor seen on PET/CT scan was due to the accumulation of neoplastic cells, and that the basis for this accumulation was a net difference in cell birth and cell death rates. You know that the cell birth rate is a function of the cell cycle time and the percentage of the population in the cell division cycle, and that cell birth is due to division of a parent cell into two similar daughter cells. You know that the daughter cells have features similar to the parent cell because of the similarities of their template DNA, and that the daughter cells are similar to each other in large part because of the semi-conservative replication of the parent cell’s template DNA. You bother to count mitoses to define grade because statisticians have correlated neoplastic cellular kinetic parameters in certain neoplasms with natural history (think untreated Burkitt’s lymphoma and follicular lymphoma) and response to chemotherapy.

It wasn’t always so. In the first half of the 19th century, one of the scholarly debates in microscopic anatomy and pathology was how cells in the various tissues came into existence. Schools of thought ranged from spontaneous generation (Aristotle on), to physicochemical crystallization within pre-existing cells (Schleiden, Schwann), to origin of new cells from pre-existing cells (Remak, Virchow). Pasteur’s goose-neck experiments of the 1850s served to disprove the 2,000-year-old concept of spontaneous generation.

It was left to Walther Flemming1 to use histochemical stains on acid-fixed fire salamander epithelia to observe and describe the phases of mitosis and cytokinesis. The studied cells were large and flat, allowing finely detailed observations with available optics. He noted that duplication of nuclei requires an intermediate step during which there is a "metamorphosis of the nuclear mass into threads" that align and separate, i.e., mitosis. He referred to this as "indirect nuclear division," and commented that he never observed "direct" nuclear division (without mitotic progression) in the plants or animals that he studied. He coined the term "chromatin" for the stained nuclear material, and the term "mitosis," combining the Greek term mit (thread) with -osis (accumulation) to describe the process of nuclear dissolution and duplication. Flemming described the phases of mitosis, drawing the compaction, alignment, and subsequent segregation of sister chromatids into daughter cells, with reversal of the compaction process in the newly formed daughter nuclei. He noted a standard progression in the mother cell nucleus from "skein" (early prophase) to "star" (metaphase) to "equatorial plate" (anaphase). He noted that this metamorphosis into threads (mitosis) is a standardized sequence of nuclear events across multiple tissue types and across multiple species, with the end result that a mononuclear cell divides into 2, and only 2, daughter cells.

He concluded that mitosis is the usual process for nuclear (and cellular) division of a mother cell into two daughter cells, commenting: "The most significant result at this point to me, consists in having retained the possibility of reducing the division process everywhere to phenomena which are fundamentally and in principle alike, and are produced by the same forces." His documentation of division of eukaryotic mother cells into two similar daughter cells supported Remak’s and Virchow’s theory that all cells are derived from other cells ("Omnis cellula e cellula").

We now know that chromatid segregation is the last step in a cell division cycle that begins with fast, coordinated replication of the entire chromosome complement using multiple origins of replication, with centromeric cohesion of sister chromatids until microtubule tensions are balanced at metaphase.2 Mother cell DNA replication leads to sister chromatid segregation into daughter cell neo-nuclei via

Milestones in Investigative Pathology, 2010 - 2015, William K. Funkhouser, MD, PhD
Copyright 2016, American Society for Investigative Pathology

11
the stepwise process of Flemming's mitosis. Repeat as able, until your telomeres wear out. Distort the process by running through your cell division cycle checkpoints and ignoring the DNA damage, and you can end up with daughter cells showing non-diploid chromosomal numbers and translocations, all now detectable once Flemming’s stars and equatorial plates could be dissected and catalogued into unique numbers and types of chromosomes for each species. In this sense, Flemming was the father of modern Cytogenetics. Once you can put numbers on human chromosomes, you can recognize by G banding that some cases of chronic myelogenous leukemia (CML) and acute lymphocytic leukemia (ALL) show reciprocal translocations between chromosomes 9 and 22. Once you recognize this physical (9;22) translocation, you can figure out that the translocation juxtaposes portions of the BCR and ABL genes into a BCR-ABL fusion gene, and you can design drugs that normalize the excessive Abl tyrosine kinase activity of its fusion protein. No Flemming, no t(9;22), no BCR-ABL fusion gene, no hyperactive Abl, no Gleevec.

The challenge for us is to preserve and protect that evolutionarily conserved stepwise cell division cycle of DNA replication and chromatid segregation from cellular mutagens that can distort the delicate cellular equilibria of P53, RB, E2F, cyclins, CDKs, CDKIs, replication origin licenses, spindle microtubules, and cohesins. We should aim for the pristine anaphases of Flemming’s fire salamanders for our own cells, and we should aim for the scientific instinct to recognize “phenomena which are fundamentally and in principle alike.” *Omnis cellula e cellula*.

References:

The first time you touch a van de Graaff generator, you’re sure you’re going to die. Anything that can arc and make your hair stand on end can’t be good for you. Then you’re taught that those millions of volts are not what kill you, it’s the amps, and van de Graaff generators don’t make enough amps to hurt you. You’re probably wondering where this is heading, but the analogy is that in ischemic heart disease, it’s not the pressure gradient (volts) that saves you, it’s the coronary artery blood flow (amps).

Those pristine coronary arteries of your youth have transmogrified over years of dedicated service into strictured, lipid-laden, thrombosis-prone vessels that reduce coronary artery blood flow. Stenotic narrowing reduces blood flow more than common sense understanding would estimate. Yes, the vessel radius and blood flow are directly related, but not linearly as with the pressure gradient, instead to the fourth power of the vessel radius. So, narrowing a coronary artery to 1/2 of its usual radius leads to reduction of blood flow to 1/16 of expected. Turbulence can develop, and could affect plaque surface stability. Therefore, atheromas in the coronaries have a marked effect on usual pulsatile laminar flow. No wonder your interventional cardiologist wants to sell you a shiny mesh stent to dilate your stenotic foci - if you double the diameter at the stenosis, you raise blood flow 16-fold. Our colleagues in Anesthesiology think this way when choosing calibers of endotracheal tubes and catheters. We Pathologists think this way because we have seen the coronary artery stenoses in association with sudden cardiac deaths from fresh myocardial infarcts (MIs), and in association with pump failure from old MIs. But it wasn’t always so.

The anatomists of the Renaissance, Andreas Vesalius (see De humani corporis fabrica libri septem, published in 1543) and his students (Gabriele Fallopio, among others), began accurate, thorough documentation of normal human anatomy. In the 18th century, Dr. Morgagni focused and published on pathologic anatomy, and as such is the father of modern anatomic pathology. His detailed case reports of gross post-mortem findings, The sites and causes of diseases investigated by anatomy (1761, when he was 80, so get to it), positioned the rest of us to see cause and effect relationships between diseased organs/cells and the patient’s signs/symptoms. The concept of disease could then evolve from that of a limited set of symptoms and signs to a vertical thought process that integrated anatomic/physiologic etiology, pathogenesis, and distortion of normal function. Signs and symptoms were a consequence of the disease process, not themselves the disease. Dr. Morgagni’s descriptions of pathologic anatomy led his intellectual offspring (including us) to see disease through this etiology-pathogenesis-altered function lens. For example, he described cardiac mural fibrosis (“the fleshy fibres of the heart themselves sometimes degenerate into a tendinous hardness”), but would leave it to future generations to sort this out microscopically and conceptually.

Dr. Edward Jenner (of cowpox vaccine fame) recognized coronary artery mural calcifications and intimal thickening in autopsy patients with a history of angina, and postulated in letters to Dr. Heberden in the 1780s that angina pectoris could be due solely to coronary artery narrowing. Dr. Parry’s clinical review of angina pectoris in 1799 credited Jenner for this coronary stenosis-angina association. However, invention of the stethoscope (Laennec, 1816) focused clinical attention on the sounds of valves and pump failure, and invention of the electrocardiogram (ECG) by Einthoven in 1895 focused clinical attention on electrical arrhythmias, such that cardiologists did not seriously revisit the correlation of coronary artery occlusion, angina pectoris, and myocardial infarction until reviewed by Dr. James Herrick in 1912. As late as 1907, a review of the works of our current Milestones...
honoree, Dr. Carl Weigert, mentioned his contributions to histopathology (e.g., myelin and elastin stains) without any reference to his 1880 description of coronary artery disease in association with myocardial infarction and mural fibrosis. We could argue that they ignored the concept because nothing could be done about ischemic heart disease at that time, but vasodilators like amyl nitrite and nitroglycerine were used from the 1870s on, and would have supported Dr. Weigert’s hypothesis regarding pathogenesis of myocardial infarction and mural fibrosis. Perhaps they were distracted by the latest diagnostic technologies, or had not been taught how to think in terms of etiology-pathogenesis-altered function, or both.

Dr. Carl Weigert was a careful student of pathologic anatomy who started out as a treating clinician in the military in 1868. His interests turned to microscopic anatomy and histochemistry in the 1870s, when specific stains for classes of macromolecules, cell types, and microorganisms were hot topics. He was folded into the specialty of Pathology and mentored by Dr. Cohnheim at Leipzig after 1874, moving to Frankfurt after Dr. Cohnheim’s death in 1884 to work with Edinger and Ehrlich. Dr. Weigert pursued the concept that coagulative necrosis was frequently the trigger for a proliferative/repairative host response. In the case of heart disease, he postulated that coronary artery occlusion led to coagulative necrosis of cardiac muscle, which in turn led to mural scarring in survivors. The title paper, written in German, is discussed in the English literature, as follows:

“In the writings of Professor Weigert and Dr. Huber, I have met for the first time with what appears to me to be an accurate and tenable explanation of the relationship existing between obstruction of the coronary arteries and the formation of fibrous patches in the walls of the heart. .....has shown that, where the circulation is slowly interfered with by sclerosis of the arteries, atrophy, with destruction of the muscular fibres, takes place without injury to the connective tissue. The shrunken fibres are thus set off by the stringy connective tissue..... If, however, the arrest of the circulation is more sudden in its onset, then yellow dry masses similar to coagulated fibrin make their appearance.....the muscular fibres and the connective tissue show no nuclei – a necrosis has occurred. .....the condition of the coronary arteries must be regarded as one of the most important etiological elements in the production of the morbid changes (cardiac mural fibrosis) at present under consideration.”

We can now expand Dr. Weigert’s working hypothesis to say that elevated serum cholesterol contributes to atheromatous plaque formation via increased serum low density lipoprotein (LDL), and that unstable plaque rupture leads to arterial thrombosis, with subsequent symptoms and signs of myocardial ischemia and possible infarction.

Because of Dr. Weigert and his articulation of this model for pathogenesis of ischemic heart disease, we can now reduce the rate of development of arterial plaque by lowering our cholesterol/LDLs with statins, we can further reduce our risk of coronary arterial luminal thrombosis with aspirin and Plavix, and we can improve our coronary artery blood flow by stenting focal stenoses. The net result is that the progression of atheroma formation is slowed, fewer unstable plaques mean fewer ruptures to trigger thrombosis, and coronary artery blood flow in stented vessels can again approach laminar flow. And even your broker can’t match Poiseuille’s return on investment – a 16-fold increase in coronary artery blood flow in return for a 2-fold increase in vessel diameter.

Because after all, it’s not the volts that kill you, it’s the amps.

References:


Milestones in Investigative Pathology, 2010 - 2015, William K. Funkhouser, MD, PhD
Copyright 2016, American Society for Investigative Pathology
Microscopes, Bacteria, and DNA: Connecting Three Centuries of Scientific Discovery From van Leeuwenhoek and Hooke to Flemming and Avery

William K. Funkhouser, MD, PhD
Originally published in ASIP Pathways, Volume 9, Issue 1 - February 2014

Scientific knowledge is like a fabric in which threads of discovery are woven both by contemporaries and over time. Parallel discovery by contemporaries is at once both competitive and synergistic. Serial discovery over time connects scientists of like instinct who have never met, yet share both discovery and indebtedness (‘standing on the shoulders of giants’, etc.).

Nowadays, we refer to the ‘thread’ of conversation, the ‘thread’ of an argument, and the ‘thread’ of a concatenated email. This Milestones’ topic threads together microscopes, bacteria, and DNA, and connects the scholarly work of 3 scientists over 3 centuries. The novel Milestones topic concerns Antonj van Leeuwenhoek and his contemporary Robert Hooke, and the thread extends through previously discussed Walther Flemming (see ASIP Pathways, Volume 7, October 2012) and Oswald Avery (see ASIP Pathways, Volume 3, February 2008).

If you can see, you can observe, measure, hypothesize, manipulate, experiment, model, understand, categorize and conclude. Since there’s a lower limit of resolution with the naked eye, magnifying lenses had to be crafted before anyone could observe microscopic organisms. Available technology (convex lenses), engineers (Hooke), and practical end-users of simple microscopes (van Leeuwenhoek) synergized to discover and document protozoa, fungi, and bacteria within the 20-year period 1660-1680.

Thread #1 is literally van Leeuwenhoek’s work as a fabric merchant, during which he used simple biconvex magnifying lenses to measure fabric thread densities. The historical narrative is that his interest in microscopic biology was triggered by Hooke’s Micrographia (1665), in which simple and compound microscopes were used to visualize plant, insect, and fungal structures. (Hooke is credited with the word ‘cells,’ an example of a word that fundamentally changed human understanding of the physical world.) We presume that van Leeuwenhoek made his own microscopes, although he didn’t publish this aspect of his work (we’re all proprietary about something). His written observations in Dutch were regularly submitted throughout his adult life for publication in English by the Royal Society of London. His competitor Hooke supported publication of van Leeuwenhoek’s work after doing confirmatory experiments in parallel. Van Leeuwenhoek is a good example of an entrepreneur without a formal background who makes and publishes critical early discoveries that lead to the development of a new field, in his case Microbiology. In letter 18, he documents a number of protozoans and bacteria in stagnant water. He was surprised at the number, variety, and mobility of these organisms, and had objective observers write on his behalf to confirm the findings of tens of thousands of organisms in a drop of rainwater. His little animals (‘animalcules’) were sufficiently well-described for subsequent biologists to name and recognize the organisms he described, so his hand-made microscopes were of good quality.

Tools for purification and experimental manipulation of bacteria allowed characterization and use. Although Simmelweiss clinically proved that something microscopic was responsible for puerperal fever in the 1840s, it required the culture techniques of Pasteur and Koch in the latter half of the 19th century to purify and speciate Streptococcus pyogenes. Likewise, although S. pneumoniae (pneumococcus) was almost certainly the old person’s friend during the 17th and 18th centuries as well, it wasn’t until the association of certain infectious agents with certain clinical presentations that medicine began to test for the etiologic role of bacteria using Koch’s postulates. Both S. pyogenes and S.
pneumoniae were isolated in culture by Pasteur in the 1880s. Culture methods were perfected for speciation, multiple types of S. pneumoniae were recognized, and antibodies were raised, allowing manipulation of different types.

Thread #2 is Walther Flemming’s 1882 publication regarding eukaryotic mitosis, derived from the Greek work mit for thread, in which he described the accumulation of chromosomes during the M phase of the cell cycle (see previous Milestones article in ASIP Pathways, Volume 7, October 2012). It was subsequently recognized that chromosomes are made of both nucleic acids and proteins, with a prevailing hypothesis in the first half of the 20th century that the chromatin proteins, rather than the nucleic acids, bore the information required to transmit hereditary characters to offspring. It wasn’t until 1944 that we understood which polymeric component of chromatin bears the template information of heredity.

S. pneumoniae evolved into an experimental model in the first half of the 20th century. Smooth/encapulated/virulent and rough/non-virulent strains were found to cause disease in mice. Griffith found that infections of mice with heat-killed virulent/smooth stains could transform the viable rough/non-virulent strain to become smooth/virulent (1928).

Thread #3 is the Avery group’s methodical biochemical dissection of the polymer responsible for this pneumococcal transformation. Avery’s group was able to purify DNA by precipitation (as threads, of course) in 100% ethanol, with demonstration of pneumococcal type transformation using DNA alone as the putative transforming agent. This work culminated in 1944’s “Studies on the chemical nature of the substance inducing transformation of pneumococcal types”, J Exp Med, 1944, 79:137 (see discussion in previous Milestones article, ASIP Pathways, Volume 3, February 2008). Avery published one paper that year, but that paper stands as an example of thorough scientific proof of cause and effect. It is DNA, not protein, which is responsible for the type transformation of rough pneumococcus to smooth pneumococcus.

Avery’s critical publication was not critically embraced by the scientific community for another 10 years. I guess ten years is better than the 200 years it took for van Leeuwenhoek’s discovery of bacteria to lead to Simmelweiss, Pasteur and Koch. It led to the base pairing studies of Chargaff (1950), the dsDNA structure solution of Watson and Crick (1953), the semi-conservative DNA synthesis experiments of Meselson and Stahl (1958), and the demonstration of the 3-base genetic code by Crick, Nirenberg and others that mapped DNA sequence to amino acid sequence (1961). Thus, allelic threads of DNA ultimately encode allelic threads of protein, explaining both DNA replication and hereditary transmission of character allelism in the population. We now know that coding sequence is a minor component of total DNA in eukaryotes, that coding sequence can be alternatively transcribed by splicing, that the steady state abundance of mRNA is regulated by both transcription rates and specific microRNA abundance, and that steady state protein abundance is regulated by both translation rates and targeted proteasomal degradation.

Perhaps the best part of this thread of a story is that some of the bacteria with which we co-exist can be put to work making human proteins for treatment of human diseases, e.g., cloned human insulin for diabetes mellitus (1981) and cloned human anti-human VEGF Ig for wet macular degeneration (2004).

Our current understanding of our genome may be akin to the earliest observations of van Leeuwenhoek about his rainwater animalcules – ‘surprising with respect to the number, variety, and mobility.’ Those static threads of our DNA may be much more dynamic than we think. Just ask Barbara McClintock.
Whistleblowers of the 1950s: Epidemiologists and the Association Between Cigarette Smoking and Increased Risk of Lung Carcinoma

William K. Funkhouser, MD, PhD

Originally published in ASIP Pathways, Volume 10, Issue 1 - January 2015

As you review your slides to find yet another new case of lung primary squamous cell carcinoma or small cell carcinoma, you may ask yourself if you are being passively complicit in an epidemic of disease caused by carcinogen-rich dusts from inhaled tobacco smoke from cheap, high-quality commercial cigarettes. We physicians and scientists are so used to treating problems and putting out fires that perhaps we have become desensitized to the notion that somebody else might be creating some of these problems, and that identification and elimination of certain etiologic agents might prevent certain diseases. This Milestones article features the 1950 publications of Wynder/Graham1 and Doll/Hill2, which present sound data pointing to inhaled cigarette smoke as the etiology of most primary lung carcinomas. A previous ASIP Pathways Milestones article3 by the first Milestones editor, Richard G. Lynch, was published in 2003 and featured the 1957 publications of Oscar Auerbach and his colleagues4,5.

Tobacco smoking in pipes had been associated with lip and oral cavity carcinoma in the 19th century6. The elucidation of cigarette smoking’s association with lung carcinomas was confounded by non-specific plain film lung radiographs (tuberculosis was common), high frequency of smoking in men, infrequency of lung carcinoma, and the lag time for development of lung carcinoma in smokers6. Now is a good time to discuss preventable cigarette smoke-associated lung carcinomas, as well as secondary diseases in affiliated non-smokers. A combination of commercial profitability, willing government subsidization, lax public health oversight, wide legal moats, addictive components in the commercial product, and individual users’ willingness to downplay the inherent risk of cigarette smoking, has led to this 100-year epidemic of acquired, preventable diseases.

Mortality due to primary lung carcinoma in men in the United States (US) in 1950 was about 20 per 100,000, at the time representing a 30-fold increase during the first half of the 20th century. The increased mortality rate due to lung carcinoma was recognized early in the 20th century (e.g., Adler8), with subsequent discussion of multiple potential etiologies, including air pollution, insecticides, arsenic, and ‘irritants’ in cigarette smoke6. The sound statistical associations between cigarette smoking and increased risk of lung carcinoma were detailed in 1950 by Drs. Wynder and Graham from the US1, and by Drs. Doll and Hill from the United Kingdom2. These statistical associations led to subsequent studies over the last 65 years that have dissected the step-wise morphologic and molecular pathogenesis of small cell and non-small cell lung carcinoma due to cigarette smoke. 100 years after the introduction of ‘Camel,’ although the biomedical research community understands much about the pathogenesis of tobacco-associated diseases, we still have not developed effective programs to extinguish the morbid and mortal diseases that are associated with inhalation of cigarette smoke.

The whistle-blowers of this preventable epidemic were Ernest Wynder and his chest surgeon collaborator, Evarts Graham, and epidemiologists Richard Doll and A. Bradford Hill. The challenge was to collect and analyze data sufficient to demonstrate a difference in lung carcinoma incidence between smokers and non-smokers at a time when >50% of men in these countries smoked.

Before Wynder, Doll, and Auerbach, there were
Henle and his protégé Koch, the anatomist and microbiologist who articulated the logical principles behind demonstration of causality in bacterial infectious disease (“Koch’s postulates”), namely:

1) The presumptive etiologic agent would be present in every case of the disease.
2) The agent would not occur in other diseases as a non-pathogen.
3) The agent can be purified.
4) The purified agent can induce the disease anew.

Strict adherence to these Henle/Koch guidelines makes sense for culturable agents like Streptococcus pyogenes and Mycobacterium tuberculosis, but it is a problem to apply these criteria to putative etiologic agents in neoplastic diseases. The papers by Wynder/Graham and Doll/Hill associated increased cigarette smoke exposure with increased risk of lung carcinoma, but multiple discoveries (DNA crystal structure, organic chemistry of cigarette smoke, DNA replication chemistry, DNA repair chemistry, DNA codon-to- amino acid translation rules, cytogenetics, distortion of signaling biochemistry by mutations of oncogenes and tumor suppressor genes, immunohistochemistry, DNA sequencing chemistry, DNA methylation measurement, copy number variation measurement, mRNA cluster analysis), were subsequently required to define the etiologic agents in the cigarette smoke, and the step-wise pathogenesis of the different types of lung carcinomas.

Alfred Evans summarized the challenges to strict interpretation of the Henle/Koch postulates as they relate to determination of etiologic agents in viral and non-infectious diseases in the Thomas Parran lecture published in the American Journal of Epidemiology in 1978. Evans recommended use of the following criteria for demonstrating causality of both infectious and chronic diseases:

1) Prevalence of the disease should be higher in those exposed than in those not exposed.
2) Exposure to the putative cause should be present more commonly in those with the disease than in those without the disease.
3) The incidence should be higher in persons who are so exposed than in those not exposed, as shown in prospective studies.
4) Exposure to the suspected factor should precede the disease.
5) There should be a measurable biologic spectrum of host responses.
6) Experimental reproduction of the disease should be demonstrated.
7) Elimination of the putative cause should decrease the incidence of the disease.
8) Prevention or modification of the host response should decrease or eliminate the expression of the disease.

Data from the articles of Wynder/Graham and Doll/Hill support several of the Evans criteria:

1) Wynder and Graham found that cigarette smoking preceded development of squamous and small cell undifferentiated lung carcinomas in 99% of (605) men.

It seems foreign to us now, but 85% of the (780) men in their “general hospital” control group without lung carcinoma smoked as well. Doll and Hill also found that cigarette smoking preceded development of lung carcinoma in 97.7% of (647) men. Similar to the US control group, 96% of the (622) men in their “non-cancer” control group smoked as well. This difference was statistically significant at p<10^-5. Both papers considered potential biases, concluded that lung carcinoma did not precede smoking habitation, and that there was no obvious etiology that could cause both smoking habitation and lung carcinoma. These data argue that cigarette smoking is an “important factor in the production of carcinoma of the lung” (Doll and Hill).

2) Wynder and Graham found that male lung carcinoma patients had higher smoke exposures than their general hospital control group (Fig. 3), p<0.0001.

Doll and Hill also found that male lung carcinoma patients had higher smoke exposures than their non-cancer control group. (Table V), p<0.001.

These data argue that higher and/or longer cigarette smoke exposure increases the risk of development of lung carcinomas. Because cigarettes are standardized doses, Proctor estimated that one new lung carcinoma is generated for every three million cigarettes smoked.

One of the challenges of assigning a direct causal relationship between a putative etiologic agent and the disease phenotype is the lag time between initial exposure (or deficiency) and subsequent disease. This is true for a variety of diseases ranging from infectious disease (e.g. tuberculosis), to neoplastic diseases (e.g. cigarette smoke associated lung carcinoma, asbestos-associated mesothelioma, and aniline dye-associated bladder carcinoma), to vitamin deficiencies. In the Wynder and Graham cohort of 605 male lung carcinoma patients, cigarette smoking histories ranged from 15 to 65 years, with a mode of 40 to 44 years; 96% had smoked for more than 20 years. In the Doll and Hill cohort, 95% of 688 male lung carcinoma patients had smoked for more than 20 years.

Wynder, Graham, Doll, and Hill are good early examples of...
epidemiologists who sought to identify preventable diseases, and work such as theirs has triggered the development of adequately powered sample sizes, new trial designs, and statistical methods for comparison of patient treatments and outcomes.

Wynder, Graham, Doll, and Hill were correct – increased cigarette consumption leads to increased incidence and mortality due to lung carcinoma. Perhaps the most telling subsequent data are the similar curves for global cigarette consumption and lung carcinoma mortality from 1900 to 2000, offset by about 30 years5. Lung carcinoma mortality in men peaked in the US in about 1990, at 4 to 5 times the mortality rate seen in the 1940s by the whistleblower authors featured in this *Milestones* article. Proctor6 estimated that 100 million deaths were attributable to lung carcinoma in the 20th century, and wondered whether the 21st century will set a new mortality record of 1 billion lung carcinoma deaths. We are currently clocking 1.6 million deaths due to lung carcinoma worldwide each year, on par with AIDS and tuberculosis.

Aside from the institutional embarrassment attendant to our scientific/medical/public health community’s unwillingness/inability to confront and eliminate cigarettes from the marketplace, these data beg the question of why humans take unnecessary risks. Slovic11 makes the point that experts and consumers estimate risk differently. Experts use statistical data to estimate risk, e.g., annual mortality rates attributable to the behavior. For example, experts would rank cigarette smoking as the 2nd riskiest behavior (1st place goes to motor vehicles).

Consumers, on the other hand, consider emotional factors like dread of catastrophe, impact on future generations, and their voluntary decision to participate, such that cigarette smoking ranks as less risky to non-experts than handguns, motor vehicles, and nuclear power. Voluntariness may be the most interesting aspect of human perception of risk. For example, a person may downplay the risk of a voluntary activity such as cigarette smoking, while exaggerating the risk of involuntary activities like consumption of perceived-hazardous food preservatives. Thus, the voluntary decision to smoke cigarettes, as well as the delayed development of the smoking-associated disease phenotypes, means that 20% of the adult US population (men and women) still consider the benefits of cigarette smoking to outweigh the risks. Don’t even get me started on the contribution of cigarette smoking to the development of emphysema, myocardial infarction, or stroke.

**References:**

8. Adler I: Primary Malignant Growths of the Lungs, and Bronchi, New York, NY, Longmans, Green and Co., 1912