Remarks for “Lifetime achievement award from Yale Cancer Center”

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GRATITUDE:

I am very surprised and thrilled to receive this award and am greatly moved by the significance of your recognition of my work carried out over 49.5 years that I have been here at Yale. My interactions with many organizations and many individuals here at Yale University School of Medicine have made my work possible. No investigator at Yale is an island; each investigator has access to an archipelago of islands within easy reach of each other. My archipelago has included Yale College, Yale Graduate School, Yale School of Medicine, Yale School of Epidemiology and Public Health, the Yale MD/PhD program and of course the Yale Cancer Center. The Yale Gym and Yale School of Music are peripheral islands that I like to visit.

For more than forty years work in my lab on Human cancer viruses has been part of a Program Project supported until this day by a grant from the National Cancer Institute of the NIH. This program project was originally formulated by Sherman Weissman and has been brilliantly led for three decades by Daniel DiMaio. My collaborators and co-workers on that grant have included Dan DiMaio, Joan Steitz, David Ward, Paul Liebowitz, Paul Sigler, Joanne Sweasy, Walther Mothes, Peter Glaser, Akiko Iwasaki, Yong Xiong. The focus has been on basic molecular mechanisms of cell transformation and tumorigenesis by viruses. The disciplines of this group have included classical virology, cellular biology, molecular biology, genetics, structural biology, immunology, DNA repair. The success of the group has depended on close interactions of investigators with expertise in a broad range of basic sciences relevant to understanding cancer etiology and pathogenesis. Communications among the project leaders and students in their labs has been greatly enhanced by the Viral Oncogenesis seminar program sponsored by the Yale Cancer Center.

I have been privileged to guide and to learn from many gifted students in my lab at Yale. These students worked as undergraduates, graduate students in basic sciences, MD/PhD students, and post-doctoral fellows. These students, the idealistic bright young people with stars in their eyes, have been the innovators, entrepreneurs and engineers of the projects.

During a sabbatical in Amsterdam in the mid 1970s I embraced molecular biology. I learned to transfer DNA into cells and to run Southern blots. I dropped many of the blots on the floor. I haven’t done any experiments with my own hands since 1990 when I went on sabbatical at Harvard to study transcriptional activation in brewers yeast. The talented and inspired students in my lab, who did not drop the blots, conceived and carried out all the experiments. All of them started out by working on

EB Virus, my passion, but as far as I know only two of them (Ren Sun at UCLA; Ayman El-Guindy here at Yale) now work on tumor virology. Most of them are involved in basic research in cancer, on breast cancer (Ron Weigel), tumor immunology (Cliona Rooney; Naomi Taylor), Stem Cell transplantation (Sara Nikiforow), multiple myeloma (Robert Orlowski) and Prostate Cancer (Srinivas Viswanathan), apoptosis (Sam Katz here at Yale). Two students have gone to the dark side but have had brilliant careers as neuroscientists working on prions (Tricia Serio) and synaptic transmission (Kelsey Martin). Virology is not a bad way to learn how to become a scientist. I am a great believer in the education and inspiration of young scientists. They will solve the many problems that remain. We need to nurture and support them at a very early age. Which leads me to tell the story of

HOW I BECAME A TUMOR VIROLOGIST:

As a second year medical student I was captivated by a lecture by an elderly visiting scientist Peyton Rous, who in 1911 discovered a virus that causes sarcoma in young chickens. It took more than 40 years for the significance of Rous' discovery to take hold; intense study of Rous Sarcoma virus ultimately led to the discovery of cellular proto- oncogenes. Among the other medical students in my class who heard Rous was Mike Bishop who shared the Nobel prize with Harold Varmus for discovery of Src, the Rous sarcoma virus oncogene. They showed that Src was derived from a cellular gene now called a proto-oncogene.

As a medical student I became fascinated by the possibility that viruses might cause cancer in man. By the late 1950s and early 1960s when I was a medical student, there were four well documented examples of viruses that caused tumors in chickens, rabbits, mice and frogs. The idea that viruses might have anything to do with human cancer had very little support; cancer viruses were thought to be limited to so called "lower animals".

As a 4th year medical student, intrigued by viruses and by naturally occurring anti-viral substances, such as interferon, I worked for four months in the laboratory of John Enders who discovered ways to propagate and to identify many different viruses in tissue culture. Enders' rather simple experiments in cell culture led to vaccines against poliomyelitis and ultimately did away with the need for iron lungs. Enders and his students succeeded in propagating mumps virus, measles virus, rubella virus, varicella/Zoster virus and cytomegalovirus. Except for cytomegalovirus there are now vaccines against these viruses.

When I was working in the Enders lab as a medical student other members of the lab were studying Simian virus SV40 a small, supposedly simple DNA virus(5200 nucleotides) that contaminated certain batches of poliovirus vaccine that were made in kidney cells from Rhesus monkeys. Enders obviously was concerned that a contaminant in poliovirus vaccine produced in monkey kidney cells might cause cancer in man. SV40 did not cause cancer in the monkeys which harbored it without detectable symptoms, but it caused tumors in Syrian Hamsters. Enders was trying

to understand why the SV40 virus was oncogenic in rodents but not in primates. I watched his experiments with intense interest but did not participate. I worked trying to develop a semi-quantitative assay for interferon, analogous to the antibiotic disc sensitivity assays for bacteria. My experiments had no demonstrable results other than convincing me that I loved working in the lab.

SV40 became the darling of molecular biologists. As a student I heard James Watson declare that if we understood SV40 we would understand cancer. In his initial tenure as director of the Cold Spring Harbor Laboratory he devoted a lot of attention and resources to SV40 and other small DNA viruses. The problem with Watson's dictum of course is that SV40 understood vastly more about the cell than Watson (or the rest of us) did. As Dan DiMaio has revealed in a brilliant recent essay the small DNA tumor viruses such as SV40 taught us an enormous amount of cellular and molecular biology. Work with SV40 led to understanding of mRNA processing and development of tools to create genetic maps of DNA, two discoveries that led to Nobel prizes.

After my rotation as a medical student, I was hooked on viruses. I undertook a residency in Internal Medicine and then I signed on as an EIS officer at the CDC in Atlanta. My job there was to conduct surveillance of cases poliomyelitis and encephalitis in the United States in order to evaluate the success or failure of the poliomyelitis vaccines. I also participated in vaccination programs against measles in Central America and West Africa. I discovered personally that by understanding viruses one could prevent disease! After clinical training and epidemiologic training at the CDC I returned to Enders' lab in 1966 as a research fellow intending to do experiments developing ways to measure cellular immunity to viruses. These experiments inadvertently kindled

MY LOVE AFFAIR WITH EPSTEIN-BARR VIRUS:

As a guide to study activation of lymphocytes engaged in cellular immunity to viruses, I studied lymphocyte activation by mitogens that caused resting cells to divide. Mitogen activated lymphocytes are good cellular targets for viruses that do not grow well in resting cells. (HIV not known at that time is a well-studied example). Along the way Enders implanted in me the idea that mitogen activated lymphocytes might be a way to grow Hepatitis viruses, long on his list of target viruses that he would like to isolate in tissue culture.

We obtained lymphocytes from leukemia patients at the Jimmy Fund who developed hepatitis after transfusion of mixed units of platelets from multiple donors. We activated the lymphocytes with a mitogen, phytohemagglutinin from kidney beans, and put the mitogen treated lymphocytes together with tissue cultures that we thought might reveal the cytopathic effects of hepatitis virus. After a few weeks in culture some lymphocytes from one leukemic child, named LS, began to proliferate extensively. Enders was familiar the work of Epstein who discovered the virus and Henle who had shown that Epstein-Barr virus could cause

normal lymphocytes to grow continuously in culture. We asked Henle for antisera that would recognize the only EB viral antigen known at the time, called viral capsid antigen. This antigen was present in a few of the LS cells, about 1%.

Through endless conversations, mostly at lunch time when Enders shared brownies from his lunch basket, we posed a series of biologic questions that I tackled in the last years of fellowship and continued for the first decade that I was at Yale. I will tell you these questions but will not try to lead you through experiments we did attempting to find the answers. For convenience I will list four main questions (I like 4 questions) : 1) Since only some of the LS lymphoid cells contained the viral capsid antigen, were the other proliferating cells from LS infected with Epstein-Barr virus? 2) If they were infected what was the virus doing in there? 3) Did the continuously growing cells make a transition between no viral capsid antigen expression (a condition we now call latency) to one in which capsid antigen and new virus particles are made? 4) Were cells caused to grow continuously by Epstein- Barr virus converted into bona fide tumor cells? The answer to all 4 questions is yes: All cells were infected by EB virus; EB virus made the cells immortal. The virus in the cells moved spontaneously and mysteriously between a state of latency to lytic gene expression. The immortal cells were tumorigenic in primates or when placed in immune-deficient mice.

I used Cotton Top marmosets (tamarins), available at the New England primate center, to test whether lymphocytes immortalized by Epstein-Barr virus caused tumors. I exposed marmoset blood cells to EB virus in test tubes . After the cells began to proliferate I put the cells back into the same marmosets that donated the cells. Some of them developed lymphomas. These lymphomas did not look like Burkitt Lymphoma the tumor where EB virus was discovered, but resembled lymphomas that were known to occur in immunosuppressed patients who had received kidney transplants.

An unanticipated and remarkable feature of the EBV infected marmoset cells was that they released large amounts of extra-cellular virus . I was later able to show that cell free virus from these cultured marmoset lymphoid cells induced lymphomas when injected back into marmosets. This was the first time that a human virus was shown to be oncogenic in primates. Once having shown that EB virus was tumorigenic we set out to

FIND EBV GENES THAT DO THE WORK OF IMMORTALIZATION, TUMORIGENESIS AND LYTIC CYCLE ACTIVATION.

A goal of many workers in the field was to map and study the function of the viral genes that were essential to these processes. Work along these lines in my lab consumed approximately four decades from 1980 to the present. Without going into details we discovered 4 viral genes whose products are essential for the capacity of EBV to change a resting cell into an immortal B cell line. We concentrated on this property because without immortalization in tissue culture there are no tumors in

animals. These genes encode proteins that are essential for plasmid replication (EBNA1) and for transcription (EBNA2) of viral and cellular genes that are essential for the immortalization process. Two viral genes that we discovered with Joan Steitz specify small non-coding RNAs (EBER1,EBER2) . We also discovered 2 viral genes (ZEBRA, RTA) that switch the virus from a latent life cycle to a lytic life cycle. These genes are important because patients who develop EBV associated cancers have increased levels of viral replication. One possible connection of lytic replication with tumorigenicity is that one of the viral genes essential for lytic EBV replication (ZEBRA) promotes DNA damage, as shown by Ruth Wang'ondu a recent MD/PhD student who will be going to St. Jude's to become a pediatric oncologist. . Ayman El-Guindy, my close colleague as a graduate student, post doc and faculty member, has recently discovered 2 genes (BGLF4 a kinase and BGLF3 a protein of unknown function) that selectively regulate the process by which EBV makes structural proteins that enable the virus to transit from cell to cell. The very large EBV genome carries about 70 genes encoding proteins and non-coding RNAs. EBV also encodes many micro RNAS. The functions of many genes has been inferred only by homology to genes that have been studied in other herpesviruses. But the eight genes that we discovered are unique to EBV and related viruses found in non-human primates. These genes are are plausible targets for anti-viral and therefore anti-tumor therapy.

LESSONS FROM INFECTIOUS DISEASES:

The people who trained with Enders were, with two exceptions, MDs. They were clinicians, epidemiologists, and bench researchers, with a primary interest in Infectious Disease. So even though I was primarily interested in viruses as oncogenic agents my search for a faculty position after fellowship focused on infectious diseases rather than oncology. I had the great good fortune to be recruited to Yale by Dorothy Horstmann an infectious disease epidemiologist who was the first woman to hold a full professorship and an endowed chair at Yale. Dorothy was a pioneer in study of the pathogenesis of polio and the efficacy of polio vaccination. She discovered that poliovirus was present in the blood and by analyzing data from Russia, proved that Sabin's vaccine worked to prevent polio.

I am not exaggerating when I say that discovery of the microbiologic etiologies of infectious disease, prevention of infectious diseases by vaccination and treatment of infectious disease with a variety of anti-microbial agents collectively represents the greatest public health achievement of the 20th century.

Lessons from Infectious Diseases provide a blueprint for understanding etiology prevention and treatment of many cancers. When I began as a fledgling tumor virologist, no one would have predicted that today we would know that there are at least 7 different and distinct kinds of viruses, each of which has many subtypes, that cause a wide range of cancers, derived from hematologic, epithelial, liver and endothelial cells. Some of these viruses, for example the papillomaviruses, persist and directly change normal cells into cancer cells. Others, such as Hepatitis B and

Hepatitis C cause liver cancer at least in part by causing liver damage and inflammation but do not always establish permanent residence in the cancer cells. So the lesson is to continue to search for associations between infectious diseases and cancer. Not only viruses; Helicobacter pylori is associated with gastric cancer; Tuberculosis with some lung cancer. I think that we will find many more important associations between microbial agents and cancer.

No one would have predicted that we would have anti-viral vaccines that prevent cervical cancer and hepatocellular cancer. In 1966 when I began my fellowship the responsible viruses had not been discovered. There are many obvious unmet needs for work in the future: the Human leukemia virus, Kaposi's sarcoma virus, Merkel's tumor papova virus, and my favorite, EB virus. The NIH is now strongly backing a program to make and test a subunit EB virus glycoprotein vaccine to prevent many types of lymphomas, nasopharyngeal cancer and gastric cancers in which EBV plays a significant role. This is a challenge because it has been difficult to show that any inactivated subunit vaccine is effective as primary immunization against any Herpesvirus. There is hope though; the recent Shingles vaccine that boosts immunity to Varicella Zoster virus following a live virus vaccination or natural infection is a subunit vaccine whose immunogenicity has been markedly enhanced by new adjuvants.

Finally, those of us who study viral life cycles at a molecular level are banking on the discovery of new anti-viral agents that will impair various stages of viral replication, viral attachment or penetration of cells, or even reverse viral latency that will lead to cancer cures. The recent dramatic success of antivirals against Hepatitis C directed at viral non-structural proteins offers promise that we will find Achilles heels in tumor viruses that prevent their replication and inhibit their ability to induce tumors.

WORK GOES ON:

There is a saying that old soldiers never die; they just fade away. Nothing could be further from the truth for old scientists like me. Peter Medawar, an immunologist who won a Nobel for understanding transplantation immunity, in his book Advice to Young Scientists, said "old scientists never reach the stage where they think that their latest idea is not novel or significant". So we continue to work.

I'd like to speak briefly about a few projects that we are currently working on in my lab. These projects are driven by curiosity not by any clear idea of where they might lead in the battle against cancer.

1) We are investigating a brand new class of antiviral drug that are also anti-epileptic drugs, including Depacote or Valproic acid and its derivatives. The discoveries were made by Derek Daigle, a grad student, Kelly Gorres, a post doc, and Sudharshan Monrahan an undergrad. These drugs are strong inhibitors of EBV and

cytomegalovirus replication at concentrations that are achieved in the blood of patients who are treated for epilepsy and mood disorders.

2) Together with Danielle Lyons, a microbiology grad student and Michael Krauthammer, a Bioinformatician formerly here at Yale Pathology, we are investigating the role of EB virus in gastric cancer. EBV is not found in every gastric cancer. However, since gastric cancer is so common, numerically gastric cancer is the most common EBV-associated cancer in the world. We study single clones of cells derived from one gastric cancer; some clones contain EB virus; some do not. Remarkably, but not surprisingly, the EBV-positive clones express cellular genes that are not expressed in the EBV-negative cells. Two cellular genes that are up-regulated in the EBV-positive clones, Cyclin D2 and the Androgen receptor, are known to be important in cancer pathogenesis. EBV may be an important co-factor in gastric cancer.

3) Kuan-ping Yu in my lab has discovered that a cellular proto-oncogene, c-Jun, is a potent cellular inhibitor of EBV DNA replication. This is an example of a well-known aspect of all virus/ cell interactions. The virus tries to take over the cell; the cell tries to stop that. We are trying to figure out how c-Jun knows to inhibit viral DNA synthesis while simultaneously promoting cellular DNA synthesis.

4) Enders' most important contribution to basic virology was his recognition of changes in cellular morphology, such as pyknosis, cell fusion, and formation of intranuclear inclusions, that resulted from viral infection. We have taken advantage of the revolutions in microscopy and development of powerful specific reagents to study these cellular changes at the ultra-structural level. Recently, Richard Park in my lab has discovered an previously unrecognized sub-nuclear organelle which we call VINORCs, virus-induced nodules on replication compartments, which plays a role in enhancing expression of viral genes and inhibiting expression of cellular genes.

HAS ANY OF PAST WORK HAD A DIRECT EFFECT ON PATIENT CARE?

You will surmise from all I have said that I strongly believe that basic laboratory research pays rich dividends in what we now call "translation". In other words basic research is not "lost in translation". I will tell you a couple of stories where basic research on EBV in my lab has paid off.

Tools for Sero-diagnosis: Once Henles had discovered that the viral capsid antigen and another viral antigen called early antigen were expressed in a few cells of some cultured Burkitt lymphoma cell lines people began sero-epidemiologic studies to learn which populations of people developed antibodies to these antigens. Early studies showed that patients with Nasopharyngeal cancer were invariably infected and their serum contained high titers of antibodies to the capsid and early antigens. But Burkitt lymphoma cell lines were unreliable sources of these antigens. Only a few cells in Burkitt lymphoma cell lines underwent the switch between latency and

lytic reactivation. Thus, there was no readily available cell system for assessing antibody responses to early antigen and viral capsid antigen that are lytic cycle proteins of EBV. We shared the B95-8 marmoset cell line with scientists over the world many of whom visited my lab in the 1970s. A large scale screening of patients with high antibody titers to EBV early and late antigens in the B95-8 cells was the basis of early detection of nasopharyngeal cancer in China. Patients with high antibody titers were subject to nasopharyngoscopy; tumors that were detected were treated with surgery and radiation. This was the first use of a viral biomarker for cancer screening.

Tools for tissue diagnosis: Michael Lerner was an MD/PhD student working in Joan Steitz's lab. They were studying ribonucleo proteins that were recognized by antibodies from Systemic Lupus erythematosis. The basic experiment was to immunoprecipitate radiolabelled cell extracts with the antinuclear antibodies and look for the RNAs that came down; these are the famous snRNPs, small nuclear ribonucleotide proteins. Michael and I were driving to a meeting at Cold Spring Harbor when he raised the possibility that some of these snRNPs could be encoded for by EB virus since they were only detected in EBV-positive cells; they could not be identified as cellular in origin. These snRPs came down with two autoantibodies from SLE patients called Sm and La . We showed, using Southern blots, tools newly acquired after my sabbatical in Holland, that two of these abundant RNAs were encoded for by EB viral DNA. These RNAs were called EBER1 and EBER2. They are the most abundant transcripts in cells that harbor latent EBV. Greg Howe, a post-doc in Joan's lab used FISH, a tool derived by Dave Ward when he was a member of the Program project, to show that they were present in the nucleus. FISH for EBERs is now the standard diagnostic tool in pathology to determine whether a tumor is associated with EBV.

Human monoclonal antibodies: As I have said EBV secreted from the B95-8 marmoset cell line efficiently infects B lymphocytes and causes them to grow indefinitely in the laboratory, a process we dubbed immortalization. The B cells synthesize and secrete antibodies. If one clones single EBV-infected B cells one now can generate a cell line synthesizing a human monoclonal antibody. James Robinson a post-doc and junior faculty colleague in my lab (now a professor at Tulane) has made human monoclonal antibodies to HIV, Lhassa Fever and Ebola virus using this strategy. Some of these antibodies are broadly neutralizing and have undergone clinical trials.

Adoptive T cell immunotherapy: EBV immortalized lymphocytes are also powerful antigen presenting cells, possibly as powerful as dendritic cells. Clio Rooney a post-doc in my lab pioneered the use of EBV transformed B cells to raise T cell populations that can be used for immunotherapy for EBV associated B cell lymphomas that occur in recipients of bone marrow transplants. Since these lymphomas are often of donor origin the strategy is to raise an EBV infected B cell line from the donor, stimulate and expand donor T cells and infuse them into the

recipient. This strategy is now being used for T cell therapy of lymphomas and lymphoproliferative disease that arise in different patient populations, such as immunosuppressed recipients of solid organ transplants, in whom the lymphomas are host in origin. Libraries of EBV transformed B cells of different histocompatibility (HLA) groups are now available. These B cells are used to expand HLA-matched T cells. When a patient develops an EBV lymphoma it is possible to just go to the freezer and pull out the T cells of the right HLA type expand them in culture and infuse them into the patient. This strategy is being commercialized.

The strategy I just described uses T cells that recognize a number of different EBV antigens that are expressed on EBV-associated lymphomas. However not all the EBV antigens are regularly expressed nor do the expanded T cells invariable recognize all the viral antigens. One invariant antigen is EBNA, a protein responsible for maintaining episomal EBV DNA genome in the cell. The conventional wisdom was that EBNA1 is not a good target for cytolytic T cells, which are usually CD8 T cells. But Sarah Nikiforow an MD/PhD student working collaboratively between my lab and that of Kim Bottomly at Yale and Christian Munz in the Steinman lab at Rockefeller made two seminal observations. First, EBNA 1 is a target for CD4 T cells; second these CD4 T cells inhibit the outgrowth of B cells infected by EBV. It is now possible to purchase HLA-matched T cells directed against EBNA1 and use them for therapy of EBV associated lymphomas.

In connection with this story, I need to emphasize one last point: we identified the EBV gene encoding EBNA1 working collaboratively with Wilma Summers under the auspices of the molecular virology program project in 1980. Work on CD4 T cells directed against EBNA1 begun in 2000 was the topic of Sara Nikiforow's thesis in 2004. Getting these findings to the state where they were of direct applicability to patients took another decade. So, basic research pays handsome dividends but one needs patience!!! AND MANY THANKS FOR YOUR PATIENCE.