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Reflection

The journey from autologous typing to SEREX, NY-ESO-1, and Cancer/Testis antigens

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It was 1980 and I was in my last year of medical school in Taiwan. Frustrated by how limited chemotherapy was at helping the terminally ill cancer patients under my care in the ward, I was fascinated by what tumor immunology might potentially offer, as there was an excitement in the air that the newly developed hybridoma technology would produce the "magic bullets" to cure cancer. This fascination brought me to the Sloan-Kettering Institute (SKI) as a Ph.D. student in the fall of 1981. Having read a review on tumor immunology by Herbert Oettgen, Lloyd Old, and Edward Boyse (1) back in Taiwan and additional papers from Dr. Old's group, I decided to seek out Dr. Old as my Ph.D. mentor. On one sunny afternoon in December 1981, I timidly strolled into his spacious office on the top floor of the Howard Building—he was the vice president of SKI at that time—and was greeted by his secretary Charlotte outside his room. While I nervously started to explain to her why she should make an appointment for this young Asian student to see her boss, Dr. Old happened to walk out of his room, apparently on his way out. Seizing the opportunity, I briefly introduced myself to him and told him that I was interested in studying tumor immunology. Dr. Old replied with a smile, "Me, too," and turned his head to tell Charlotte to make an appointment for me to see him. That was my first encounter with Dr. Old, who surprised me that afternoon with his kindness toward a young Taiwanese student, a great character of his that I would observe again and again for the next three decades without fail.

After that initial encounter and a few following late afternoon meetings around 6:00 p.m., I officially became his Ph.D. student. Hybridoma technology had been invented a few years before that time, and Dr. Old's immunology lab was feverishly involved in making hybridoma antibodies against cell surface antigens toward many different tumor types, including melanoma, renal cell carcinoma, lung cancer, leukemia, etc., and I thought that I would be assigned to one of those projects, characterizing human tumor antigens as defined by mouse monoclonal antibodies. To my surprise, Dr. Old decided that molecular biology would be the most powerful tool in tumor immunology, and he assigned me to work with Yuichi Obata (a research associate in the lab then) to clone thymus-leukemia (TL) antigen genes from the BALB/c mice (DO NOT spell this strain as "Balb/c," as Dr. Old hated it when people did that) and establish a molecular biology minilab within his laboratory. With his connections, he sent Yuichi to Stanley Nathenson's lab at the Albert Einstein College of Medicine to learn DNA extraction, Southern blots, and transfection; sent me to Jeffrey Ravetch's lab (in SKI at that time) to learn RNA extraction and cDNA library construction (for cloning insulin receptor!); and sent both of us to IntelliGenetics in Palo Alto, California, to learn gene sequence analysis programs, at a time when GenBank was not well established and openly accessible to the public. His foresight turned out to be absolutely correct, as molecular cloning would, in a decade and later, lead to the identification of the most important tumor antigens today, including the MAGE-A antigens by Thierry Boon's group (2) and the cloning of NY-ESO-1 and other Cancer/Testis antigens such as CT7, CT10, etc., by us working with his group.

For his entire career, Dr. Old had been relentlessly involved in the discovery of human tumor antigens. He firmly believed in the existence of tumor-specific antigens and absolutely despised the term "tumor-associated antigen." As a serologist, his first major effort to identify human tumor antigens was autologous typing, a strategy designed to identify cell surface tumor antigens. As was beautifully summarized in his Clowes Memorial Lecture in 1981 (3), this strategy utilized the sera from cancer patients and tested directly against the cell lines established from the autologous tumor. The specificity of any serological reactivity detected was evaluated by absorption analysis, to see if preincubation of the sera with other cell lines of autologous or allogeneic origin would absorb out the serum reactivity. An extensive series of autologous typing experiments as carried out in his lab in several tumor types, leading to his description of three classes (class I, II, and III) of tumor antigens, class I and class II being "individually unique antigens" and "shared tumor antigens," respectively (3). These experiments confirmed his belief that tumor-specific antigens exist in human cancer and that these antigens are spontaneously immunogenic. The molecular identification of the nature of these tumor antigens, however, would have to wait another decade.

Following the same principle of autologous typing, Dr. Boon's group at the Brussels Branch of the Ludwig Institute for Cancer Research, of which Dr. Old was at the time the CEO and director, identified MAGE-1 (later renamed MAGE-A1) as the first human tumor antigen recognized by cytotoxic T lymphocytes (CTLs) in 1991 (2). This milestone achievement ushered in the era of antigen-specific cancer vaccines and ignited an intense interest to identify additional immunogenic human tumor antigens. While excited by the *MAGE* family genes and their potential as cancer vaccine targets, Dr. Old was concerned that the transfection assay-based methodology utilized for the MAGE-1 discovery had two intrinsic limitations. One was the requirement of establishing cytotoxic T cell lines from these tumor patients, and the other was the need to establish tumor cell lines from the autologous tumor specimens. Knowing how difficult (if not impossible) it is to establish tumor

cell lines from some of the most common cancer types, breast cancer and prostate cancer in particular, Dr. Old worried that it would be nearly impossible to apply the same technology to many epithelial cancers and he pondered how this could be circumvented. As a serologist, it was only natural that he again turned to serology, wondering how one might utilize humoral immune responses, i.e., autologous antibodies instead of CTLs, to molecularly clone tumor antigens that are spontaneously immunogenic in cancer patients.

This idea was borne out in 1995 by the study of Sahin *et al.* (4) at the University of Saarland in Germany, led by Michael Pfreundschuh. In their study, they constructed recombinant cDNA phage expression libraries and immunoscreened with the autologous patient sera. This methodology was termed SEREX, for "serological analysis of recombinant cDNA expression libraries" of human tumors with autologous serum (also see reflection by Dr. Pfreundschuh in this issue). In their initial application of the method to melanoma, several tumor antigens were identified, including MAGE-A antigens and tyrosinase, which were originally defined by Boon's group as T cell-recognized epitopes. These findings proved Dr. Old's concept that serology could be used to identify immunogenic tumor antigens, including intracellular tumor antigens recognized by CTLs; Dr. Old was absolutely elated.

In Dr. Old's mind, the next obvious step was to screen as many tumor specimens and as many tumor types as possible. Having that goal in mind, he sent Matthew Scanlan and me to Dr. Pfreundschuh's lab for two weeks in the summer of 1995 to learn the SEREX technique from Ugur Sahin and Özlem Türeci. One major technical hurdle that we encountered in SEREX was the need to eliminate antibodies in the human sera that would react with bacterial or phage components, as such contaminating antibodies would completely obscure the detection of antibodies against tumor antigens. The solution, as it turned out, was the old trick of absorption analysis that Dr. Old had used for autologous typing, except that in this case, the serum would be repeatedly absorbed with bacterial and phage lysates to remove the antimicrobial antibodies.

Equipped with the SEREX technique, we started our first SEREX library screening in late 1995, on a case of esophageal squamous cell carcinoma. Eight antigens made their way through the tertiary screening and were sequenced. Four of them were found to have no homologous sequences in the GenBank. Among these four, one showed normal mRNA expression only in testis, and yet was detected in several melanoma cell lines and a proportion of other types of tumors tested. This distribution pattern, although peculiar, was not unfamiliar, as it was the exact same expression pattern of *MAGE-A*, *BAGE*, and *GAGE* genes identified by Boon's group, as well as the *SSX2* gene (HOM-MEL-40 clone) identified by SEREX by Pfreundschuh's group. We knew right away that we had found a new tumor antigen, and NY-ESO-1 was born (5).

But why choose to name this gene "*NY-ESO-1*"? The explanation was simple. As I was analyzing esophageal cancer in the laboratory, Dr. Old had continued to pursue his goal of applying SEREX to as many tumor types as soon as possible. To that end, he assembled a SEREX collaborative group in 1996 through the Ludwig Institute for Cancer Research, and this group started to perform SEREX worldwide (University of Saarland [Homburg, Germany], Ludwig Institute Branches in New York, Melbourne, and London, Aichi Cancer Center [Japan], Krankenhaus Nordwest [Frankfurt, Germany], and Moscow State University [Russia]), analyzing melanoma, lung cancer, renal cancer, colon cancer, gastric cancer, etc., simultaneously. This resulted in the identification of 2,593 sequences derived from 2,169 clones (as of 2004 when this group became inactive), including CT7, SOX, ZIC2, NY-BR-1, Rab35, and p53, among others. When the researchers from this SEREX collaborative group met to present and discuss their data, the SEREX-identified clones from the individual labs were listed based on the location of the research group and the tumor type that was analyzed; in this particular case, NY(New York)-ESO(esophageal cancer)-1.

As Dr. Old and I sat down to write the NY-ESO-1 manuscript in early 1997 (5), we sensed that it was not a coincidence that MAGE, GAGE, SSX2, and NY-ESO-1 all shared the same characteristics of restricted expression in cancer and in normal testis, and Dr. Old insisted that we should come up with a new name for this group of antigens, as they likely share other biological characteristics and should be grouped together. (Not surprisingly, he was correct, as we subsequently found that many of the genes linked to these antigens were multigene families on chromosome X and had the tendency to be expressed in high-grade aggressive tumors, among other common features.) But what should we name this group of antigens? Until then, MAGE, BAGE, and GAGE had been referred to as "shared tumor antigens," distinguishing them from antigens that are either lineage-specific (e.g., melanocyte differentiation antigens) or individual tumor-specific (e.g., mutated p53). Dr. Old was not happy with the name, feeling that it was too broad a definition and too vague. We considered "cancer/germ cell antigens" but we did not know at that time whether these antigens were expressed in the ovary. (It turns out that they are expressed in fetal ovaries but are mostly turned off in adult life.) So we continued to ponder. And then one day, during one of his typical early morning phone calls to me that many colleagues must have similarly received, he said, "You know what? We should call them 'Cancer/Testis antigens, CT antigens'. That's it. CT antigens!" So the term CT antigen was born, a term that we first introduced in the NY-ESO-1 paper (5) and that today generates 2,239 results in a PubMed search (February 29, 2012).

The discovery of CT antigens as an important category of tumor antigens immediately led Dr. Old and myself to the idea that one should perform SEREX using serum samples from patients with known anti-CT immune responses—either antibody or CTL—against CT-rich cDNA libraries, e.g., testicular cDNA library and CT-rich cell line libraries. This strategy proved to be successful, leading to the discovery of CT7 from the SK-MEL-37 cell line library (6) and SCP1 by Pfreundschuh's group (7). Multiple previously defined CT antigens—*NY-ESO-1*, *LAGE-1*, *MAGE-A*, and *SSX* genes—were also isolated in these and subsequent experiments, reconfirming their capability to elicit antibody responses (6).

The next task was to prove that these SEREX-defined CT antigens could elicit not only humoral but also cell-mediated immune responses. NY-ESO-1 turned out to be the prototype example of this dual immunogenicity, but that would be a story best told by Alexander Knuth and Elke Jäger (see their joint reflection in this issue).

While the immunologists in Dr. Old's lab—Elisabeth (Lisa) Stockert and Sacha Gnjatic—and the collaborating groups in Europe—Alexander Knuth, Elke Jäger, Danila Valmori, etc.—were busy analyzing the antibody, CD8, and, later, CD4 responses to NY-ESO-1, MAGE-A1, MAGE-A3, and other tumor antigens, the molecular biologists in Dr. Old's lab—Matt Scanlan, Ali Güre, and myself—shifted our CT identification strategy from SEREX to molecular-based methodology. By representational difference analysis and comparing mRNA from

melanoma versus normal skin, a MAGE-A-related CT gene, *CT10*, was cloned by Ali Güre (8), and anti-CT10 antibody was demonstrated by Dr. Stockert in a melanoma patient, establishing its immunogenicity. The introduction of massively parallel signature sequencing (MPSS) by Andrew Simpson (LICR-Saõ Paulo) to the LICR-New York colleagues brought this comparison of mRNA expression to a depth that was previously unimaginable, and >20 CT or CT-like genes were identified by applying this new technique to testis, melanoma cell lines, and other somatic tissues, most significantly *CT45* (9), a gene subsequently shown to have a prominent expression in Hodgkin lymphoma. In addition to these experimental approaches, *in silico* analysis was simultaneously employed as a tool to identify new CT antigen genes, a task that involved Victor Jongeneel's group in LICR-Lausanne. *CT46* was identified by analyzing the EST (expressed sequence tag) databases for genes with Cancer/Testis-restricted expression (10). *CT47* was discovered by analyzing previously unknown multigene families on chromosome X (11). These three latest CT genes, *CT45*, *CT46*, and *CT47*, referred to by Dr. Old as the CT-trio, would represent the last wave of CT antigen discovery in Dr. Old's laboratory. The final push that Dr. Old spearheaded for CT antigen discovery was the comprehensive analysis of the mRNA expression data at a genomic level by Oliver Hofmann and Winston Hide that would involve all available data using a combination of four platforms: MPSS, ESTs, CAGE, and RT-PCR (12). This thorough analysis resulted in the cataloguing of a total of 153 genes with mRNA expression in normal tissues restricted to, or at least preferentially in, testis, with evidence of tumor expression. Otavia Caballero in the lab then went on to experimentally evaluate possible new CT antigen genes on this list, but no new CT antigens with promising cancer expression profiles were identified. We then concluded with confidence that we had exhausted the pool of the CT antigens, and this chapter of CT antigen discovery in Dr. Old's laboratory was closed.

As the ending note to the CT chapter, Dr. Old decided that he would like to create an electronic list in which he could just scroll down and see all information on every CT antigen, from their genomic organization, mRNA expression, and protein expression, to their immunogenicity in clinical trials. He called this the CTpedia—a database in which all our knowledge on CT antigens would be deposited and organized. For this task, he enlisted the help of LICR-Saõ Paulo, namely Ana Tereza Vasconcelos and their colleagues, including Andy Simpson and Otavia Caballero who had by that time moved to work in LICR-New York, and CTpedia was established in 2009 (13, 14). This database now includes 138 CT genes or gene families, reflecting all antigens that have been published to date as CT antigens in the literature.

Almost exactly 30 years passed from the afternoon that I first walked into Dr. Old's office in 1981 to the time that he passed away, and he was my mentor, collaborator, and a great friend for all three decades. As someone who truly enjoys travel, I believe this long winding road of tumor antigen discovery that I have explored with Dr. Old as the guide by my side has certainly been the most incredible, or, using one of Dr. Old's favorite words, "fascinating" journey of a lifetime. Thank you, Dr. Old.

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