

A man of vision and the discovery of tumor necrosis factor

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This is a personal reflection about Lloyd Old, an outstanding scientist and extraordinary man, as seen through the eyes of these authors, who worked with him for over 40 years. Our perspective is unique in that we shared a very close relationship with him during the formative years of his career, from 1958 when he arrived at Sloan-Kettering Institute (SKI) at the age of 24, until we retired from the Ludwig Institute for Cancer Research (LICR) about 10 years ago. Lloyd was a brilliant man whose life's work contributed immeasurably to science, and his personal strength of character touched the lives of all those who knew him.

Lloyd began his scientific career in the summer of 1958 at a small lab bench in the Division of Experimental Chemotherapy at SKI. He had just graduated from medical school in California and drove east to New York City in his new, bright red Corvette to begin a research fellowship. Those of us who were technicians in the department were young, single females, so there was immediate curiosity about this man who was also single, handsome, and polite. Because of Lloyd's interest in immunology, he began to collaborate with Dr. Baruj Benacerraf at New York University (NYU). During his first weeks, none of us got to know anything more about him or even to speak with him except on a few occasions when passing in the hallway. He worked by himself and was frequently downtown at NYU, so there was little opportunity for interaction. When in his lab, he seemed very serious about his work and, unfortunately, wasn't particularly interested in chatting with us. Before long, he needed mice for experiments, and the cages were kept in a large stock/experimental room where we spent most of the day. Once the mice arrived, he would 'visit' them almost on a daily basis to check on their well-being. If he discovered a cage was dirty or a water bottle had leaked onto the shavings, he was visibly infuriated. This always ended with an angry encounter between him and the animal maintenance staff who were immediately called into the room. The unpleasantness of these encounters spilled over, involving any of us who were unlucky enough to be there at the time. After this occurred several times, a couple of us decided to keep an eye on his mice, and if anything was amiss, we would take care of it first thing each morning, knowing that Lloyd was not an early riser. This worked very well for everyone concerned. For many, many years afterward, those who worked in his lab had the late day chore of checking every animal cage before going home. For Lloyd, only the highest standards were acceptable, and this applied not only to how experimental animals were cared for and treated, but also to how research was to be conducted.

In the spring of 1959, the construction of SKI's Walker Laboratory, located in Rye, New York, was completed, and the Division of Experimental Chemotherapy, among others, relocated there. This was especially advantageous for Lloyd because now he had his own lab, ample animal quarters (which were sorely lacking in NYC), and permanent technical assistance.

The sudden death of Dr. Cornelius P. Rhoads, director of SKI, resulted in a major downsizing of experimental chemotherapy research, thus additional technical staff was invited to transfer to Lloyd's lab—among them Elisabeth Stockert, from Austria, and Gayla Geering. Elisabeth remained in the United States and in our lab until her death from cancer in 2002. She became a close personal friend of Lloyd and a major contributor to Lloyd's research legacy. Gayla also made major contributions before entering veterinary school in 1971. Dr. Ted Boyse joined us in 1961 and began a long collaboration with Lloyd; graduate students started training with us, and the first postdoctoral fellows arrived from Europe and Japan.

It's of interest to speculate on how Lloyd's career might have developed if it weren't for Walker Laboratory and the opportunity to expand the scope of his research so rapidly. He was bursting with ideas, and looking back to these years, one can see how successful his research was by reviewing his bibliography of the early 1960s.

Bacillus Calmette-Guérin (BCG) and other stimulators of the reticuloendothelial system (RES) and their effects on tumor growth interested him greatly. The study of the antigenicity of carcinogen-induced fibrosarcomas and leukemia in inbred mice, made possible by our newly developed mouse colony, was a huge project that lasted several years and eventually included inbred guinea pigs and rats. He was fascinated by Dr. William B. Coley's promising studies using bacterial toxins in human cancer patients. Through his friendship with Helen Coley Nauts, who had kept her father's work alive despite the discredit it had received, Lloyd became an outspoken advocate of the potential clinical role of these toxins. Thus, endotoxin from *E. coli* became an important part of our lab's anti-cancer repertoire. We observed the dramatic tumor hemorrhagic necrotizing effects of endotoxin on carcinogen-induced fibrosarcoma cell lines in mice. (At this time, the extensively studied BALB/c Meth A cell line, induced with 3-methylcholanthrene, was developed and converted from a solid subcutaneous tumor into a more convenient ascites form. Meth A played a critical role in the discovery of tumor necrosis factor [TNF].)

Murine viruses, causing spontaneous mammary tumors and leukemia in some strains of mice, were valuable models for *in vivo* experiments, as well as for studying cell surface antigens, using serological methods that had been improved by Ted and Elisabeth. Murine tumor cells were grown in tissue culture to establish permanent cell lines for *in vitro* studies. The era of L-

asparaginase also began in the early 1960s, and experiments expanding on the initial reports about its anti-cancer properties became a major focus of our lab. The guinea pig, already a celebrated research animal because of its gift of serum complement, so necessary for the serologist, became an even greater star, along with its larger relatives from South America. You might be startled by the presence of an agouti or capybara watching nervously from its cage when you walked into the office shared by Lloyd and Ted.

Lloyd was more relaxed these days. He truly liked mice and enjoyed lifting off the lid of a cage, talking gibberish to them, and allowing them to run over his hands. He was surrounded by a group of friendly and enthusiastic co-workers, and it seemed he had moved beyond the anxieties shown at the beginning of his career. The lab staff was quite young, or at least young at heart, energetic, and fun-loving. Lloyd's sense of humor and playfulness became evident, and he gave nicknames to some of us, names that stuck forever. Those of us who were technicians and without graduate degrees were treated as equals in Lloyd's eyes, a reflection of his self-confidence, as well as of his confidence in us. He gave credit where credit was due, as evidenced by the order of authors in many of the lab's publications. He was full of ambition and had boundless energy. Close friendships developed and we became his 'family,' especially important to him because he never married or had a family of his own. He was kind, courteous, and devoted to us. He expected complete loyalty from us, and through the years, those who strayed from his interpretation of loyalty found him difficult. During the many years of his career, despite a hectic schedule, he gave generously of his time to anyone who needed his help or advice.

Working with Lloyd in the early years and being trained by him was not always easy or altogether pleasant, but he taught us very well. He stood over us as we performed experiments, literally breathing down our necks. We were taught to be compulsively accurate and never sloppy or haphazard. We were pushed to do more and more, and his ideas kept coming. Eventually, either we got very frustrated, or we learned how to make him back off. We'd no sooner launch into some experiment that he wanted done right away when he'd come up with another idea, expecting results on this latest project practically immediately. It was a bit of a game for him too; he would walk into the lab and hound us for results, knowing very well that there were none. We usually made note of his latest ideas and dealt with them eventually, or possibly forgot them. In later years, his staff referred to this as Lloyd's 'flavor of the week.' He never stopped thinking about the science. We were expected to work long hours, and when 'vacation time' was mentioned, we were told that 'there is so much to do,' i.e., how could we even conceive of taking a vacation. He instilled a sense of guilt in us about taking time off, but as years went by, he was forced to give up these tactics when we stopped 'asking' but, instead, 'announced' that we would be going away. After many years, he relented and began taking some time off. He pushed himself very hard, year after year, but eventually he realized that rest was necessary for the mind and body. Of course, some of us found it entertaining to jokingly criticize him for his absence.

As Lloyd's confidence in our abilities grew, we worked mostly on our own, and he encouraged us to be creative. By necessity, we gradually but steadily discouraged his continual presence in the lab, which quite frankly slowed down our progress. He had taught us how to think 'out of the box,' and with his amazing memory and knack for synthesizing bits and pieces of information from everywhere, he could create a new concept or

direction of research. This ability served him well as his career moved into major administrative roles where directing research was on a much larger scale. He thought that much more could be accomplished when groups worked together in a structured way, from a central core, rather than working totally independently.

In 1962, SKI presented Lloyd with the Sloan Award, which offered him a year of advanced study at institutions around the world. Although he felt honored by receiving this award, those who knew him well were not surprised when he asked to delay his absence from the lab for a year. So much was going on that leaving was unthinkable. He managed to put it off until being promoted to associate member and moving us to the new Kettering Laboratory in NYC in 1964. Reluctantly, Lloyd left New York for several months to fulfill his obligation to SKI and the stipulations of the Sloan Award. No longer having to commute by car from his NYC apartment to the Rye lab may have been fortunate for more than his career. Lloyd drove the Corvette aggressively, and he was not a defensive driver. Following several mishaps which miraculously caused no serious injury, he gave up the car. We knew this was a very good idea.

In the late 1960s, Lloyd's lab staff grew as more fellows and graduate students arrived. Although much of the serology focused on the mouse and other laboratory animals, there was a gradual shift toward studying human cancer antigens, especially as primary cancers and sera from patients became more accessible and tissue culture methods improved for the development of human cancer cell lines, including Burkitt's lymphoma. Now Lloyd had his sights set on the difficult task of discovering human cancer cell antigens and developing immunological means for treating patients.

Naturally occurring murine viruses causing spontaneous cancers in some strains of inbred mice were studied in greater depth. Gross virus caused spontaneous leukemia in adult mice of the AKR strain. It was fortuitous that Dr. Bob Kassel at Columbia University began to collaborate with us to study these leukemic mice. We observed that injecting serum from mice of other strains caused regression of the AKR disease, at least temporarily. Because we knew that BCG and endotoxin had anti-cancer effects, we decided to collect serum from normal mice treated with one or both of these agents in hopes of enhancing this 'normal serum' effect in the leukemic mice. Also, because the leukemia was caused by a virus, we wanted to treat the mice with interferon, but only species-specific human interferon was available. Other researchers had reported that endotoxin induced interferon in mice and that BCG-treated mice were unusually sensitive to the lethal effects of endotoxin, even at very low doses. We decided to inject normal mice with BCG, and then with a low dose of endotoxin on day 14, hoping interferon would be present in the blood when the mice were bled two hours later. Within 30 minutes, the mice began showing signs of shock, and some died before two hours.

Lloyd contacted a friend who could perform *in vitro* murine interferon assays using L cells, a murine fibroblast cell line. The results were astounding. The serum couldn't be assayed properly because it killed the L cells. Control experiments showed that endotoxin itself did not kill L cells, nor did the serum from normal mice injected with endotoxin or with BCG. In his great wisdom, Lloyd said, "Inject some of that L cell-toxic serum into Meth A mice." This meant to inject normal BALB/c mice with cells from the Meth A tumor cell line to obtain rapidly growing subcutaneous tumors, and then at day 7 inject the 'toxic' serum intravenously into these mice and see what happens. As noted previously, sometimes his ideas were put aside or forgotten by

the recipient, but in this case, and very fortunately, it was remembered and tested. Rapidly growing Meth A tumors never responded to any anti-cancer treatments with the exception of endotoxin, which caused tumor hemorrhagic necrosis but severe toxicity. When day 7 arrived, the mice were injected with the 'toxic' serum, then put to bed for the night.

On the following day, the day that TNF was discovered, the mice were alive and well, but their round, fleshy tumors of the previous day were flat and black! There was nothing remaining except a black scab where the tumor had been. Lloyd was summoned to the animal room to see this apparent miracle, and he was amazed. What had caused this? Thus began four years of inquiry into proving or disproving that the phenomenon was caused by residual endotoxin, interferon, a combination of the two, or possibly something else. The answers didn't come easily or quickly, and Lloyd Old never published unless he was totally convinced that the results were correct. He never wanted to be in a situation where his publication had to be retracted.

A lot of work had to be done, and Lloyd contacted labs, both within SKI and at other institutions, who could help us. Large pools of the 'BCG plus endotoxin' serum were collected from mice. Serum was partially purified by chromatography, and fractions were tested for hemorrhagic necrosis in Meth A-bearing mice, as well as for interferon and endotoxin content. An interferon-free fraction causing tumor necrosis still killed L cells but not normal cells, and endotoxin was absent as shown by Limulus amoebocyte lysate (LAL) assays and rabbit pyrogenicity tests. Finally, it was clear that neither residual endotoxin nor interferon were the cause of tumor necrosis *in vivo* and L cell cytotoxicity *in vitro*. The discovery paper was published in the *Proceedings of the National Academy of Sciences* in September 1975; the name given to this previously unidentified protein, tumor necrosis factor (TNF), was chosen because of its amazing tumor-damaging capability.

During the latter 1970s, murine TNF serum was produced in large quantities, substantially purified by multi-step chromatography, studied *in vivo* and *in vitro* in our lab, and shared with other investigators. A human cancer cell line was discovered that produced TNF, and an extensive panel of human cancer cell lines was tested for murine and human TNF sensitivity; TNF was not species-specific, unlike interferon.

It became evident that TNF played a major role in inflammation and immunity, but genetic sequencing of both murine and human TNF by Genentech and others in the mid-1980s was the crucial step toward greater understanding of this amazing molecule. Today, TNF research is in the hands of investigators around the world, but its discovery was due to a brilliant scientist and treasured friend, Lloyd Old.

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