In memoriam: César Milstein, who with the late Georges Köhler invented monoclonal antibodies, died on 24 March 2002. Their invention sprang from basic research on antibody diversity and specificity, and spawned revolutionary advances in biology, medicine and industry.

César Milstein, the father of modern immunology

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César Milstein was born in 1927 at Bahia Blanca, Argentina, to immigrants from Russia involved in the secular, intellectual Jewish culture of the time. César was an adventurous youth who went to college in Buenos Aires, where he majored in chemistry and was active in politics. It was through politics that he met his lifelong love, Celia, and after graduation and marriage, the couple hitchhiked through Europe on a year-long honeymoon.

After returning, César carried out enzyme research under Stoppani for his Doctor en Química degree at the Universidad de Buenos Aires, while he and Celia scraped together just enough money to support themselves by moonlighting as clinical biochemists. In 1958, César received a prestigious British Council fellowship and sailed with Celia to England. Milstein published papers on kinetics on his own and on the amino acid sequence of enzyme active sites with Fred Sanger and received a second Ph.D. degree in 1960. Sanger had completed the amino acid sequences of the insulin A and B chains in 1951 and 1953 and the assignment of their intrachain and interchain disulfide bonds in 1955. Milstein helped Sanger celebrate his first Nobel prize in 1958. The Milsteins returned to Buenos Aires in 1961, Celia to complete her Ph.D. studies and César to what appeared to be a promising position as head of the Division of Molecular Biology at the Instituto Nacional de Microbiología. However, funding was cut off after a military coup and an ensuing vendetta against liberals and intellectuals at the institute. Finding the situation impossible, César wrote to Fred Sanger. In 1963, César joined Sanger and others under the chairmanship of Max Perutz at the recently created Medical Research Council (MRC) Laboratory of Molecular Biology.

Celia and César Milstein, with Tim and Noah Springer, in front of the Fogg Art Museum at Harvard, 1992.

The generation of antibody diversity

Milstein has cited both a research student he inherited in Argentina who was working on antibodies, and the suggestion of Fred Sanger, as stimuli for his decision to work on the antigen-combining sites of antibodies. Shortly after Milstein's death, Sanger with typical modesty denied his own involvement in this decision and noted simply that antibody diversity was a hot topic at the time. Rodney Porter was undoubtedy another influence. Porter had already begun studies on antibodies in 1946 as Sanger's first Ph.D. student, and the Porters and Milsteins later became lifelong friends, taking annual walking vacations in Europe. In 1958 Porter had described the antibody Fab and Fc fragments, and in 1959 Gerald Edelman described dissociation into heavy and light chains, enabling the modern view of antibodies as Y-shaped molecules with two Fab fragments and one Fc fragment to emerge in the early 1960s. The question of whether antibody diversity was a consequence of sequence variation had become a soluble problem.

Milstein first approached this problem by determining the sequence of disulfide-bonded peptides in Bence-Jones light chains and obtained evidence for both variable and constant sequences. Milstein also defined the inter-heavy chain disulfide bridges that characterize each immunoglobulin (Ig) subclass. Milstein became an advocate of somatic mutation and, with Sydney Brenner, published a paper on this topic in 1966. As is often the case, the advocates of the opposing schools of germline diversity and somatic mutation both turned out to be right, with a combined mechanism far more complex than imagined by anyone.

César's interest in the mechanism of somatic mutation drove his research for the rest of his life and was the impetus that led him to invent monoclonal antibodies. It is a tribute to Milstein's taste and instincts that of all the mechanisms for antibody diversification, somatic mutation continues to be the most enduring research problem and the most relevant to the issue of affinity maturation. It is a tribute to his enthusiasm and tenacity that he contributed a paper on this topic the very week before he died.

As a scientist, César was open, approachable and loved to discuss scientific issues. His early activity in politics seemed to have developed his ability to see a problem from all possible angles, gnawing on it until the best approaches for solving it were found and carried out, and then clearly arguing all the evidence in support of a conclusion. He was interested in the big picture, but also in the smallest detail that could shed light on it. Furthermore, he was not interested in an approach if others were taking it, but sought unique ways of attacking scientific problems.

From myelomas to hybridomas

In the 1970s, César turned from the sequencing of myeloma proteins to mRNA sequencing and work with myeloma cell lines (antibody secreting tumors) cultured *in vitro*. RNA sequencing required huge amounts of cells and ^{32}P. When I first met him in 1977, much of his

time was spent with myeloma and hybridoma cells, and I was impressed that they were almost like children to him. Cells were maintained in spinner flasks out in the lab, with ingenious methods for drawing off exponentially growing cells when needed for experiments and for continuous infusion of media. With George Brownlee in 1972, César translated mRNA from the myeloma cells, discovering a signal sequence on the light chain precursor. Their sequencing efforts also revealed in the mRNA the junction between the variable and constant regions, which showed that their joining preceded protein synthesis. The development of recombinant DNA and DNA sequencing opened up new approaches, but they were taken by many others, including Terry Rabbitts, whom César had hired at the MRC. Therefore, in characteristic fashion, César decided to embark on two new approaches, and it was the combination of these that was to lead to monoclonals.

With David Secher and Richard Cotton, Milstein searched for somatic mutations in the cultured cells. This required both prolonged culture to allow mutations to accumulate and the screening of thousands of clones. The first examples of mutations in somatic cells were identified, but the mutations were not in the variable region and thus were not the type sought.

Thwarted in their investigation of hypermutation, Cotton and Milstein decided to use the myeloma cells to study the basis for allelic exclusion. Myeloma cells with distinctive IgGs were selected to be susceptible to different drugs and fused together. Somatic cell hybrids were selected based on their drug resistance. The hybrids secreted Ig types from both myeloma parents. Thus, allelic exclusion was not dominant. Furthermore, the V (variable) and C (constant) regions of the two different Igs did not become intermixed, suggesting that joining had occurred at the DNA, rather than the RNA, level.

The first monoclonals

Georges Köhler came to Milstein's lab to continue the work on somatic mutation, with the idea of screening for mutations in the antigen-combining site of myeloma IgGs using antigens. However, only a few myelomas with the antibody-like ability to bind haptens were known, and they failed to grow in the lab. Frustrated, Köhler and Milstein hit upon the idea of combining the two projects in the lab on myeloma cell fusion and somatic mutation. They would make their own antibody-secreting cells by fusing myeloma cells to lymphocytes from animals immunized with a specific antigen. In the resulting hybrid cells, the immune lymphocyte parent contributed the specific antibody heavy and light chains and drug resistance, whereas the myeloma cell parent contributed immortality and the phenotype of prodigious Ig secretion. Fortuitously, the two keys to experimental success—techniques for cell fusion and robust, drug-susceptible myeloma cells—had already been established in the lab. The very first experiments succeeded, and the "hybridoma" revolution was born.

A paper entitled "Continuous cultures of fused cells secreting antibody of predefined specificity" was published in *Nature* in 1975. The initial antibodies were to sheep red blood cells, an immunogen with the practical advantage that clones of antibody-secreting hybridomas could be directly visualized as plaques in lawns of red blood cells. However, the technology would soon be extended to every conceivable immunogen. Furthermore, the experiments were successful beyond Köhler and Milstein's wildest dreams. Approximately 10% of the hybridomas secreted specific antibody, whereas plaque-forming cells secreting specific antibody constituted only about 0.1% of the white blood cells in immunized spleens. The lymphocytes that were dividing because they were responding to the specific antigen turned out to be particularly fusogenic. This 100-fold bonus in the frequency of specific hybridomas was termed by Milstein "the gift of God".

Despite this resounding success, other labs did not immediately adopt the new technique. Milstein actually worried that the technique might die out and resolved to establish it firmly. Giovanni Galfré joined the lab, optimized hybridization and made it reliable by substituting polyethylene glycol for Sendai virus as the fusing agent. A period of remarkable productivity followed, in which Milstein and Galfré fused, grew and cloned hybridomas, and collaborators in other labs provided the immunized animals and carried out the assays for specific monoclonal antibodies. In the process, the enormous potential of the method was realized, and the hybridoma revolution spread around the world.

Fruits of the revolution

At the time, cell surface proteins were thought to be of enormous importance, but they were in a lipid bilayer "swamp" that hindered work on their identity or function. The true strength of the monoclonal approach was that incredibly complex mixtures, such as whole cells, could be used for immunization. The only "purification step" required to obtain monospecific antibody was to clone the hybridomas. Huge arrays of previously unknown molecules on the cell surface were soon to emerge. One of the early enthusiasts was the late, irrepressible Alan Williams. Alan was fond of diagrams that showed monoclonal antibodies at the center of all cell surface research. Together with Milstein and Galfré, the first monoclonal antibody specific for a T lymphocyte subset was produced, to rat CD4. Len Herzenberg, who had just brought fluorescence-activated cell sorting to biology was on sabbatical in the Milstein lab in 1976, and the synergy between sorting and monoclonals immediately became apparent. With Jonathan Howard, the first allospecific monoclonal antibodies were raised to the rat MHC. With Andrew McMichael, the first monoclonal antibody was obtained to a human leukocyte differentiation antigen, CD1. César would continue to work on CD1 for the rest of his career. With Claudio Cuello, César collaborated on the immunocytochemistry of the nervous system. Herman Waldmann would go on sabbatical with Milstein in 1978 and make antibodies to human hematopoietic cells. One of these would be called CAMPATH-1 for Cambridge Pathology, would be "humanized" with Greg Winter at the MRC lab, and would eventually be approved as a therapeutic for chronic lymphoblastic leukemia.

Monoclonals illuminate T cell biology

Many fields were transformed by monoclonals. I will recount their impact on T lymphocyte biology, which I witnessed directly. I had gone to Cambridge in 1976 to pursue the holy grail of how T lymphocytes recognized antigen. It seemed impossible at the time to do molecular work with whole T cells; however, attention-grabbing work was being done on "soluble, antigen-specific T cell factors", which were reported to be responsible for all types of cellular immune functions. After half a year, the antigen-specific factor I was working on turned out not to exist. It was the most crucial turning point in my career when I met with César in early 1977 and asked to work with him. We planned to make rat monoclonal antibodies to mouse T lymphocytes. Milstein, Galfré, Secher and I made antibodies to mouse differentiation antigens, including one to the integrin Mac-1 on macrophages. Later, I used monoclonal antibodies to directly screen for functionally important molecules in cellular immunity and identified adhesion receptors of the immune system.

My experience mirrored what was going on in T cell immunology as a whole. Not only were monoclonal antibodies used to define the many receptors and coreceptors of T cells, and even the antigen-combining

site itself, but also an analogous approach emerged of making antigen-specific T cell hybridomas. The mysteries of T cells were unlocked and, ironically, it was César's work on B cells that provided the key.

A cornucopia of antibodies

By the early 1980s, studies on cell surface molecules had resulted in so many antibodies and names that a Babel of terminology emerged. Alain Bernard and Laurence Boumsell organized a monumental exchange of antibodies and data, and a common language, cluster of differentiation (CD). A council was organized to approve nomenclature and ensure the continuity of the workshops, with César as president. As the number of CD antigens and antibodies grew, the work became enormous, with literally over 100,000 aliquots of antibodies changing hands in workshops. The strain on the organizing laboratories was enormous. After each workshop, it appeared that no one might be willing to organize the next, even bigger one. Here César's political skills truly shone. Each time he was the force behind the scenes, cajoling or arm-twisting to ensure the next workshop. His global political vision ensured eight workshops on four different continents and the largest exchange of reagents ever organized for basic and clinical research.

Despite Césars efforts, monoclonal antibodies were never patented. Prime Minister Margaret Thatcher, a chemist herself, was reported to have criticized this bureaucratic "failure" under her predecessor's watch at an impromptu seminar at 10 Downing Street. The indirect financial benefits for the UK were nevertheless large, and the benefits to humanity enormous. The use of monoclonals is as pervasive in the pharmaceutical industry as in academia. Monoclonal antibodies are used extensively in diagnostics, even in everyday applications such as home pregnancy test kits. Furthermore, many monoclonal antibodies have been approved as drugs and have benefited over a million patients with cancer, rheumatoid arthritis and heart disease.

Later in César's career, he continued to make important contributions to the study of monoclonal antibodies, somatic mutation and CD1. In a series of very elegant studies, he used monoclonal antibodies as "snapshots" of the somatic mutations that occurred during the affinity maturation of the oligoclonal immune response to haptens and showed that on-rate as well as affinity was important in maturation. César was active in advancing science all over the world, particularly in Latin countries, and served on many international and foreign funding agencies.

A scientist and a nice chap

César achieved greatness with a laboratory of modest size. His students were a privileged few who cherished his scientific wisdom, generosity and charm. César's invention of hybridomas as well as his enthusiasm, openness and fair dealings with others allowed him to attract many outside collaborators and rapidly spread his methods and ideas. César's colleagues at the MRC were equally fortunate. He recruited several who worked independently, but sometimes published with him, and will carry on his tradition. Greg Winter grafted the complementarity determining regions of rodent monoclonal antibodies onto human frameworks, and thus "humanized" antibodies for compatibility as therapeutics. He also invented a new generation of man-made antibodies that bypass animal immunization and allow expression and selection in bacteria and phage. Michael Neuberger is uncovering the molecular basis of immunoglobulin somatic hypermutation, gene conversion and class switching.

César's contributions to science and mankind were recognized with almost every prize imaginable, including the Nobel Prize, the Copley Medal of the Royal Society, the first MRC Millennium Prize and a Companion of Honour for services to molecular biology.

César leaves behind his greatest love in life, Celia. They were an unusually close couple who shared many interests in life. Celia, who is also an immunologist, was on the staff at the Babraham Institute, and the couple published together and met regularly at their home for lunch. On vacations they would often go with friends on walking tours, stopping at a different village each night.

Among César's other loves were gourmet food, fine wine, English and Spanish literature, music, theater, arguing a point on almost any subject over Cognac or Chateau d'Yquem and walking with friends and his lurcher dogs. Despite adhering to a strict diet during his 25-year battle with heart disease, he was able to nimbly adapt his gastronomy. He was a master in the kitchen, and paella was his signature dish. Allusions to food even found their way into his talks, particularly the after dinner variety. He referred to monoclonals as "antibodies *á la carte*," with each course ordered exactly to taste and appearing on its own dish. With due apologies that he had no intentions to offend his audience, he then said that the mixture of antibodies in serum was analogous to the waiter arriving at the table with the appetizer, entrée, salad and dessert all floating with the soup and wine in a single tureen.