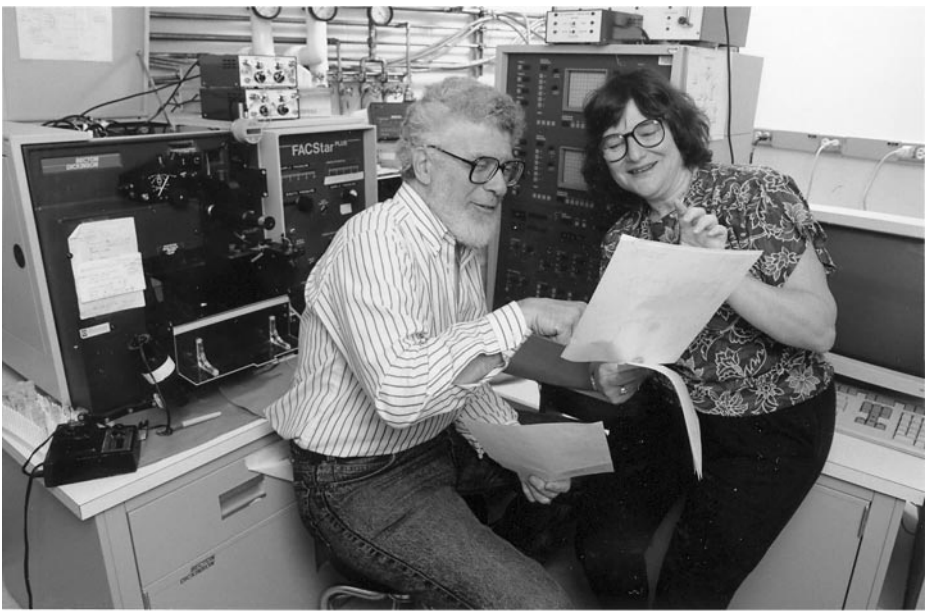


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# GENETICS, FACS, IMMUNOLOGY, AND REDOX: A Tale of Two Lives Intertwined

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## INTRODUCTION

We (Len and Lee Herzenberg) have worked separately and together for more than 50 years. This blending of independence and mutual reliance is reflected here as we shift back and forth in telling the story of the laboratory we have led and the life we have lived. The space provided for this chapter is very generous. Yet, calculated out, it amounts to roughly 100 words per year for each of us. To make the most of this, we have written an autobiography rather than a history. In many instances, we have referred only briefly, or not at all, to work that had major influences on our thinking. In addition, we have adopted a policy of naming the many students, fellows, and collaborators with whom we have worked only by referring to our joint work with them. We hope the reader realizes there would be no biography worth writing were it not for the contributions made by these and all of our other colleagues.

## THE CALTECH YEARS

LEN: There were nine professors in genetics in the California Institute of Technology (Caltech) Biology Division when I arrived in 1952 as an entering graduate student. They worked with different organisms and taught different areas of biology, but they were united by a common theme—how genes are expressed and how they influence the appearance, physiology, function, and behavior of the organism.

The Biology Division at that time was small—one three-story building housed the entire faculty, about a dozen postdocs, an equal number of graduate students, and a couple of undergraduate biology majors. Among the roughly 20 faculty members (visiting or permanent), there were seven Nobelists in the making: George Beadle, Max Delbruck, Ed Lewis, Renato Dulbecco, Roger Sperry, James Watson, and Barbara McClintock. Linus Pauling, Caltech Chemistry chair and winner of

two Nobel prizes, was in the next building, connected to ours by a much-used corridor. Creative thinking and challenging discussion were the rule; research productivity was the outcome.

The development of tools and techniques that removed barriers to experimentation also played a central role in the Caltech Biology Division culture. We routinely used the pH meter and DU spectrophotometer recently invented by former Caltech chemistry professor Arnold Beckman. My thesis advisor, H. K. Mitchell, was an extraordinary glass blower and tinkerer who worked with me on an electrophoresis device. Most things couldn't be bought, so we had to build them. As I think about it now, the automated fly counter that Ed Lewis built probably sowed the seeds for the Fluorescence-Activated Cell Sorter (FACS) that I developed some years later.

In the same way, looking back at the cross-discipline culture in the Caltech Biology Division, I see the origins of the eclectic research goals that Lee and I have pursued. Over the years, we have ranged broadly and drawn our students and fellows into immunology studies as diverse as showing that H-2 antigens are surface proteins, using immunoglobulin (Ig) allotypes and classical genetics to define the Ig heavy chain (IgH) chromosome region, demonstrating IgH allelic and haplotype exclusion in B cells, defining functional subsets of T and B human and murine lymphocytes, cloning and sequencing lymphocyte surface markers, identifying fetal cells in maternal circulation, understanding redox influences on transcription factor activation, and doing clinical studies to characterize and treat the glutathione deficiency in HIV infection. However, the twin themes of genetics and somatic cell function that guided (and still guide) this work, and the love of reading and talking about these diverse areas with people of different scientific interests, are well rooted in Caltech tradition.

Political activism was also important at Caltech. Joseph McCarthy, the Senator from Wisconsin who made a career of finding communists under every bed, was threatening to disrupt academic and personal freedom. In response, we joined Linus Pauling, Matt Messelson, George Streisinger, and Arthur Galston, as well as other faculty, students, and fellows in establishing a Federation of American Scientists chapter and in protesting this "witch hunt." A portion of Lee's and my life ever since has been devoted to helping the United States be the kind of country we want our children and children's children to grow up in.

LEE: When Len left Brooklyn for Caltech, he was 21 and I was 17. Logically, because he had three years of graduate work ahead of him and I had three years to finish my undergraduate degree, we decided that we would marry when we both finished school. However, logic couldn't overrule the three-thousand-mile distance, the loneliness, or the $3/minute (1953 dollars) the telephone company charged young lovers just to say hello. By the end of the fall of 1952, Len urged me to apply to schools near Caltech, and when I was admitted to Pomona College in Claremont we set a wedding date for the coming summer.

Needless to say, our parents thought we were too young, too innocent, too poor, and too crazy. They were probably right. But we got married anyway, with their

blessings, and set off on an adventure that is as exciting today as it was the day we piled our stuff into the car Len's parents gave us and started the long drive across the country to Pasadena.

At Caltech, I was as enchanted as Len was by how immersed people were in their work. I had already selected biology as my major, but I had no idea how interesting it could be. For the rest of the summer, until the fall term started at Pomona, I went to the lab every day with Len. I attended seminars; I read in the library; I learned how to help Len with experiments; sometimes I even had the courage to ask questions of someone other than Len. It was just plain fun, and I couldn't get enough of it.

Pomona College, on the other hand, turned out to be a disappointment. It was an excellent school. There is no question about that. The liberal arts courses were wonderful. But the Biology Department was still teaching gram stains (to identify bacteria) and grilling us on the anatomy of flowers and reptiles. Meanwhile, Jim Watson had just brought the double helix back to Caltech and was teaching about it while I was sitting in a classroom learning things useful only to a stodgy high-school biology teacher.

I would have enrolled as a Caltech undergraduate, but women weren't even admitted to Caltech graduate programs (there were only a few women postdocs and research associates). Nevertheless, the biology faculty believed that women were educable and worth educating. By the beginning of the second semester of the academic year, they worked out an auditing program for me in which I would be treated, and graded, like a Caltech biology major. They allowed me to take whatever courses I wanted to, and even found a part-time job for me so that Len and I could afford to eat. For each course I completed, the professor gave me a letter certifying that I had met the course requirements and received a grade (always As, as it turned out). So, although I didn't get formal credit, I managed to take classes such as virology from Max Delbruck, bacteriology from Renato Dulbecco, and immunology from Ray Owen. I learned how to think about science from these teachers.

Perhaps the most formative event for me during our time at Caltech, though, was a dinner with Barbara McClintock, who had recently come to the Biology Department as a revered visiting professor. Len and I had decided to drive into Los Angeles for a Chinese meal and were about to leave when we noticed that the only light left on in the building was coming from Dr. McClintock's door. We peeked in and saw that she was working alone. Then we drew back into the shadows, debating whether we should tiptoe away and not interrupt her, or whether she might actually like to take a break and join us. Finally, we screwed up our courage and asked her if she would like to go. "I'd be delighted," she replied, and off we went.

At dinner, I naively asked Dr. McClintock how she made such wonderful discoveries. Her answer, simple and straightforward, became my long-standing rudder. She said that in the course of her work, she occasionally got a surprising result that could not be reconciled with existing theory. First, she would decide whether she believed the "exception"—in other words, she could not see any technical or

interpretive flaws that undermined it. Next, if she believed it, she would commit it to memory and compare it with any other exceptions she had come across. Ultimately, a constellation of exceptions would coalesce to yield a testable hypothesis that, if validated by additional experimentation, would provide the basis for extending or altering the current paradigm.

Len and I have never forgotten that dinner with Dr. McClintock. Somehow, it must have been meaningful for her as well. When I next met her, some ten years later, I introduced myself by name and started to say, "Dr. McClintock, you may not remember me. . ." when she cut me off with, "Oh, you're the people who took me to that Chinese restaurant in L.A.," and she greeted me thus ever after.

## THE PARIS YEARS

Len defended his thesis in August 1955. We left immediately for Paris, where Len had organized a postdoctoral fellowship with Jacques Monod at the Pasteur Institute. My childhood friend who had recently returned to her native France met us at the boat, found us a room in a student hotel in the Parisian Latin Quarter, and introduced us to the life poor students lived in Paris. It was great! And so was the laboratory at Pasteur.

LEN: Jacques Monod's laboratory at Pasteur was physically separate but intellectually allied with Andre Lwoff's laboratory two floors above. Francois Jacob, the third member of the trio later awarded the Nobel Prize for their seminal molecular biology studies, worked in the Lwoff laboratory. The two laboratories lunched together virtually every day at a single long table in a small atrium on an inner Institute courtyard, where huge glass vessels rumored to have been used by Louis Pasteur were stored.

Lunch was marvelous. It wasn't a formal seminar, but conversation revolved around science in the lab and the world at large. Findings were analyzed, theories debated, visitors questioned. Every day was an intellectual feast.

There were also many first-person history lessons about the days before World War II and what the war was like in France. Monod was a major figure in the resistance against the Nazis. Francois Jacob had been to Algeria and North Africa and participated with the Free French in the liberation of France. Georges Cohen, one of the Monod senior scientists and still a close friend today, survived the war as a Jew in France and talked about things he did in the Resistance. We heard stories about how the laboratory hid Jewish scientists when the SS came knocking. It all sounded very romantic in 1955, ten years after the war ended. But as Georges and Jacques Monod often reminded us, it wasn't much fun when it was happening.

I should mention that, like the Caltech Biology Division, the group at Pasteur accepted Lee as an unofficial student. At the beginning, she was pregnant with our first child and spent most of her time working with me. Later, she brought the baby to the lab most afternoons and continued working.

## The Cradle of Molecular Biology

This was a very exciting time at Pasteur. The characteristics of the $\beta$-galactosidase (LacZ) operon were unfolding before our eyes as each new piece of work was completed. Under Monod's leadership, I contributed two pieces to the puzzle: I showed that the galactoside-concentrating mechanism encoded by the permease gene in the LacZ operon increases the internal concentration of inducers that upregulate expression of the LacZ operon genes and thus is responsible for the autocatalytic increase in LacZ induction. In addition, I showed that the inducers are acetylated, rather than phosphorylated (as people had thought), during LacZ induction, thus opening the way to adding (after I left Pasteur) the $\beta$-galactoside acetylase gene to the LacZ operon. These and other findings led Monod, Jacob, and Lwoff to the discovery of the LacZ operon, which laid the groundwork for much of modern molecular biology. For this, they received the Nobel Prize.

While at Pasteur, I met Melvin Cohen, who later became a Salk Institute immunologist. Mel had worked with Monod and returned to visit several times. Conversations with him then, as always, were highly stimulating. His presence in the Stanford Biochemistry Department was a key motivation in my decision to move to Stanford when the opportunity arose several years after I left Pasteur.

LEE: Aside from birthing a baby and learning to balance being a mom with being a scientist (albeit only a budding one), I don't have too much to show for my time at Pasteur. I did do one independent piece of work, but it wasn't well received. Monod had several times said that the thiogalactosides that we used to induce expression of LacZ operon genes were unnatural compounds that could not be digested by bacteria. This didn't seem right to "wise guy" me. So I went out and scooped up some fresh Parisian soil, put it into a flask with minimal medium containing thiogalactosides as the only carbon source, and put the flask into the cabinet under the bench.

About a week later, the medium in the flask was cloudy, and a clear sulfur smell wafted out when I opened the top. Something was clearly growing and "knew how" to break down thiogalactosides. Excitedly waving the flask, I went to Jacques' office to show him my prize. He was quite surprised and, in a manner I hope I have learned, graciously said he was pleased to be wrong in this case. Some time later, however, everyone in the laboratory was ready to kill me. While we never had to sterilize thiogalactoside stock solutions before my little experiment, after I opened Pandora's flask, all of the thiogalactoside stocks got contaminated. From then on, they all had to be sterilized immediately after they were made!

This incident aside, I mainly spent my time at Pasteur helping Len. Because I was rather sedentary during the first year, and because Len loved hands-on experimentation, I took over much of the data recording, computation, and display (plotting) that was needed. The work was tedious (slide rules were the closest thing to computers at the time). However, it gave me the opportunity to do a preliminary analysis of the data and try novel approaches to analyzing LacZ induction kinetics. Len left this to me. He was more interested in developing methods and

experiment designs that would enable clear conclusions without a lot of mathematical interference. This division of labor, which reflects Len's innate preference for concreteness and my innate love for theory, remains with us even today.

## THE NIH YEARS

Just about the time that Len and I were considering what to do after Pasteur, an ominous postcard caught up with us. It had spent rather a long time traveling to France by surface mail, and it announced that Len should have reported for active duty in the U.S. Army several days before the postcard was loaded onto the slowest boat on the Atlantic. Late or not, the postcard made it quite clear that Len had been drafted!

We immediately went to Jacques Monod for advice. He was as adamant as we were that it would be a pity to interrupt Len's scientific career to serve in a peacetime army. "Why don't you consider going to the National Institutes of Health, my boy? I have just had an inquiry from Harry Eagle looking for a fellow for his laboratory. He should be able to arrange for you to serve in the Public Health Service instead."

LEN: This was a shock, but I was not displeased with the idea of going to Harry Eagle's laboratory. I had already been thinking about doing genetic studies with mammalian somatic cells. What better place to learn how to grow cells than the laboratory that had just developed Eagle's medium? Leaving Pasteur and the *Escherichia coli* world would not be easy. But the challenges presented by mammalian studies would also be exciting. So, without further ado, I decided that I was lucky to have the opportunity to carry a pipette rather than a gun for my country and asked Jacques to write to Harry Eagle on my behalf.

It took several months to untangle the draft board and Public Health Service mess and to wrap up my work in Paris. But by the summer of 1957, Lee, Berri (our toddler), and I were settled in the Bethesda area, and I began work at the NIH.

Eagle's laboratory operated with more of a top-down structure than Pasteur and lacked some of the intellectual and scientific excitement I was used to. However, my colleagues in the laboratory, notably Robert DeMars (now at the University of Wisconsin) and James Darnell (now at Rockefeller University), were great.

"Captain Harry," as we sometimes called Harry Eagle (to his face as well as behind his back), was focused on determining the nutritional conditions necessary to establish and maintain long-term cell lines. I was, too, because to do mutation and selection studies I needed to establish conditions that would allow individual clones to grow. My finding that adding pyruvate to Eagle's medium was sufficient to support clonal growth let me begin exploring drug resistance markers for genetic studies. In addition, it led to the addition of pyruvate as a normal constituent of the medium.

The Federation of American Scientists came back into our lives shortly after we arrived in Washington, because the national office was only a short distance

from the NIH. Picking up where we left off at Caltech, we volunteered to work on the newsletter and do some administrative work for the organization. Ultimately, we helped to reorganize the office and put the administrative oversight into the hands of a liberal Washington, DC, law firm.

## Lee and the *Salmonella* Histidine Operon

LEE: A few weeks after we arrived at the NIH, Len ran into Bruce Ames, a Caltech buddy who had just been appointed to a permanent staff position at the NIH. As luck would have it, Bruce was looking for his first technician and I was looking for my first job. Len made the match.

Poor Bruce. No one should have had to put up with me as a technician. I was always asking "why" and looking for better ways to do things. As a graduate student, I would probably have been fine. But as a technician responsible for doing work that someone else gave me to do, and generating data that someone else was supposed to interpret, I was clearly a pain in the neck. Nevertheless, Bruce put up with me, and I learned to get the work done. I owe him a great deal.

Bruce was working on the characterization of the enzymes in the histidine synthesis pathway in *Salmonella*. We had completed the work on three of the enzymes when Bruce left for a month to work in Arthur Kornberg's department in St. Louis. As he went out the door, he handed me a tube containing the substrate for the last enzyme in the pathway and asked me to characterize that enzyme as we had the others. The only problem was that when I took the spectrum of the substrate, I found that its synthesis had gone wrong. I had no substrate to work with.

Long-distance telephone calls, at that time, were very expensive. It was unthinkable to try to call Bruce and ask for instructions. So I took the question to one of the senior investigators in our department. "Find something useful to do. Bruce will be home soon," he responded.

I cogitated over this for a bit and then decided to apply some of the operon thinking I learned in Paris to the histidine pathway. I tried out some conditions I thought would reveal coordinated regulation of the expression of the enzymes that were already characterized and, to my surprise, readily found such conditions. Bruce was pleased with this finding when he returned, but he put it aside until the entire pathway was properly characterized. After a couple of months, I left Bruce's lab because I was getting along in my second pregnancy. Bruce later completed the operon study with Barbara Garry. He included me as an author on the paper, which became my first peer-reviewed publication (1).

# THE STANFORD YEARS

LEN: The opportunity to move to Stanford came as a complete surprise. Joshua Lederberg visited Harry Eagle's laboratory toward the end of my required Public Health Service "hitch." We had a long discussion about the future of mammalian somatic cell genetics and the progress I had made thus far in developing useful

markers for the cell lines I had chosen. I was a bit preoccupied at the time because I was in the midst of negotiating a permanent position at the NIH. However, the talk with Josh was really a delight.

Later in the week, Harry Eagle called me into his office and suggested that I delay a bit before making any commitments at the NIH. I didn't make the connection with Josh's visit and was somewhat mystified. However, a few days later, a letter arrived inviting me to consider a faculty position in the Genetics Department that Josh was in the midst of establishing at Stanford!

Our parents considered the offer a disaster. California was still a long, long way away from Brooklyn, and they now had two grandchildren they wanted to help raise. Lee and I were also somewhat negative about returning to the West Coast. However, just after the offer came, we made a trip to New York that reset our direction.

We came to New York so I could attend the annual Federation of American Scientists meeting. Martin Kamen also attended the meeting and wound up walking with me at its close from upper Manhattan to the Times Square subway station. I had met Martin when I was at Caltech, at a benefit party Linus Pauling gave to help him raise funds for his legal fees. He was fighting the INS decision to revoke his passport (another McCarthy victim). We talked a bit about this, and then I told him about the possibility of going to Stanford in Josh's new department. By the end of the walk, I realized that going to Stanford was a chance of a lifetime and that there was no way I could turn down a position there, if I got it.

Why was Stanford so exciting? Well, with urging from Henry Kaplan, head of Radiology at the Stanford Medical School and the pioneering developer of treatment protocols for Hodgkins disease, Stanford President Wallace Sterling had mustered the resources to upgrade the Medical School to a first-rate institution with the twin goals of forefront research and excellent clinical practice. Arthur Kornberg, who would win the Nobel Prize in 1959, was recruited as chairman of Biochemistry, and he in turn recruited the cream of the department he chaired in St. Louis. Joshua Lederberg, who couldn't interview me until February (1959) because he had a date with the Nobel Prize in December 1958, was brought in as chairman of Genetics.

I was the first faculty member Josh recruited. Josh's (first) wife Esther was also in the department. One reason she and Josh had chosen Stanford was that, unlike Berkeley and many other schools at the time, there were no nepotism rules at Stanford that prevented her from working with Josh. I noted this, although Lee planned to look for a job in another department or possibly go back to school to get the degree(s) she wanted.

In September 1959, the Medical School began moving into its new building, which housed both the basic sciences and the hospital. Lee and I (and our two children) arrived just as this was happening. The Biochemistry Department was already in its quarters. Space had been opened for Josh's lab and the Genetics office, but little else was ready. The landscaping had not even been started, so the building stood in the middle of a hot, dusty field.

I was given temporary laboratory space in the nearby Applied Physics building and set up my lab so that I could get the cultures that I had shipped from the NIH growing. But California weather was not kind. September that year turned out to be mercilessly hot, and the building I was in didn't have air conditioning. As it turned out, it would have been better if my incubator had had a water cooled, rather than a water heated, jacket! Fortunately, I was able to recover from frozen stocks much of what I had lost.

Lee began working with me around this time. I had already applied for and gotten a grant to support my somatic cell genetics work. The funding was available, but the delays in completion of the Genetics space put everything else into chaos. I had made a list of equipment I wanted purchased before I arrived, but none of it had been ordered. The Genetics office was overworked and understaffed, and my cultures were cooking in the incubator. Lee decided to pitch in for a while to help me get started. Best decision we ever made!

Stanford was great for another reason. During McCarthy times, the University of California and many other schools required faculty to sign a loyalty oath swearing that they were not now, and had never been, a member of the Communist Party or any other organization that advocated the overthrow of the federal government. Because the list of proscribed organizations was created at the whim of people who rose to power ferreting out supposed communists, its sweep was extremely broad. Many faculty members found the requirement of a loyalty oath repugnant and refused to sign.

Stanford supported this view by refusing to institute a loyalty oath and by hiring people who left other institutions rather than sign such an oath. A number of eminent Berkeley physicists moved en masse from the Berkeley to the Stanford Physics Department. We were pleased to have the opportunity to meet and work with these physicists in the years that followed.

## We Become Immunologists

Shortly before coming to Stanford, Josh Lederberg had spent some time in Australia with Sir MacFarlane Burnet, who was head of the Hall Institute in Melbourne. Josh and Sir Mac applied genetic thinking to the immune response and came up with the idea that antibody responses reflect the clonal selection of cells that are individually committed to producing antibodies that recognize, and are triggered by, the immunizing antigen.

This so-called clonal selection theory predicted that individual cells would make antibodies specific for a single antigen and stood in opposition to instructive theories that predicted much more plasticity for individual cells. The clonal selection hypothesis ultimately won out. However, at the time Lee and I arrived at Stanford, the jury was still out.

To do the studies that would test this hypothesis, Josh appointed two young visiting faculty members: Gustav Nossal, who later followed Burnet as head of the Hall Institute, and Olli Makela, who later returned to Finland to do immunology

research and eventually became Dean of the Medical School in Helsinki. Gus and Olli, and the mouse facility they would need, were to be housed in the same corridor as the lab being completed for my use. So the move to the new building, which occurred about December 1959, put my somatic cell genetics group right next to one of the most exciting immunology projects of the time.

## The First Immunology Studies

LEE: By the time we came to Stanford, Len had already developed an interest in the practical side of immunology. Just before leaving the NIH, he arranged to visit George Snell at Bar Harbor to discuss the idea of using mouse histocompatibility antigens, rather than drug sensitivity, as somatic cell markers in cultured cell lines. Len thought it would be neat to use cytotoxic antibodies to the H-2 antigen (thought to be a single entity at the time, now recognized as the MHC) to select cell surface antigen variants in lymphocyte and other cell lines. However, he wondered whether this would be practical. So he went to talk to George, who was very encouraging and offered some antibodies for this purpose in case Len needed them.

This idea lay fallow until we sorted out all the problems involved in getting the lab set up. However, once this was accomplished, Len suggested to me that I take on the job of anti-H-2 antisera so that we would have our own reagents with which to select variants. Of course, I had never touched a mouse and knew nothing about how to proceed other than what I could read. Nevertheless, I took on the job. Len and I both liked it as a project for me because I could work independently at my own speed without creating for him a bottleneck on a critical path.

To figure out how to start, I went knocking on Gus and Olli's door. They did indeed know how to proceed, and they showed me how to take out spleens, use spleen cells to immunize the mice, and do tail bleeds to collect the sera. They weren't much help in setting up the erythrocyte agglutination assays that were used at the time to titer the sera. However, with their and Len's advice and several quite good papers on the subject, I managed to get a test going and learn to reliably read it.

There was already a great deal of serologic evidence characterizing the genetically distinct H-2 antigens expressed by various mouse strains. Because C57BL mice were known to make strong antibodies to the DBA/2 H-2, and because both kinds of mice were available from a local commercial breeder, I chose this combination. In addition, because female C57BL mice that had been retired from the breeding colony were large and could be obtained quite cheaply, I chose these mice to immunize. I got very good responses and was able to collect lots of good antisera that Len could use for selection.

Surprisingly, however, some of the control sera that I took from the breeders before immunization turned out to have low but clearly positive levels of antibodies that agglutinated DBA/2 rbc but were clearly negative against the serum producer strain (C57BL). A bit of detective work soon showed that many of the retired breeders that we had purchased had been out crossed to DBA/2 to make F1 animals

that were in high demand. Thus, by following my nose, I had discovered a potential model for human Rh immunization during pregnancy (2).

Some time later, I showed these data to Ray Owen, the Caltech professor who taught the immunogenetics course I had taken. Ray shocked me by asking when I was going to publish it. I stammered and stuttered a bit until Ray finally said, "Well, if you are serious about being a scientist, then I guess you have to publish this." So I did. For this and many other reasons, I often refer to Ray as the closest I ever had to a graduate professor.

## Focus on H-2 Antigens

LEN: With a plentiful supply of anti-H-2 antisera, I decided to phase out my drug-resistance work and focus on using these sera for genetic studies with mouse cell lines. First, however, I needed to do some characterization of the H-2 antigen, at least to the point where I knew what it was. There was general confusion on this at the time. Immunologic evidence had located H-2 on the cell surface of many cell types. However, while some people thought the antigen was composed of carbohydrate or protein, no lesser a light than Peter Medawar, who would later be awarded the Nobel Prize for discovering adaptive immune tolerance, thought that H-2 was made of DNA. We soon laid this issue to rest by isolating plasma membranes and characterizing the H-2 antigen(s) associated with the membranes as a protein or glycoprotein (3).

Working on H-2 drew me ever closer to the immunology community at Stanford. Gus and Olli became close friends as well as wonderful colleagues who loved discussing science as much as I did. Avrion Mitchison, who later headed a productive Immunology Department at University College, London, was also appointed as a visiting professor by Josh and began occupying the lab next door within the year. Together, we established an immunology journal club, which met one evening a week at my house as a no-holds-barred discussion in which we examined methodology, evaluated experiment design, questioned conclusions, and argued theory. The descendant of this journal club still functions in our laboratory today, with much the same rules.

Gus and Olli were highly focused on testing the clonal selection theory (4) during this time. Their approach was to isolate individual antibody-producing cells and determine whether a single cell made antibodies to one or both of a pair of immunizing antigens. Their data, although limited by the number of cells they could isolate and test, clearly favored clonal selection. Mel Cohn, in the Biochemistry Department three floors up, with colleagues Lennox and Attardi at other institutions, were holding down the instructive corner of the argument.

At the time all this was happening, I didn't have a notion that I would one day develop an instrument (the FACS) that would make it possible to resolve this question. However, once we got the FACS running, we returned to these issues in studies that became a major focus of our laboratory for several years.

## Enter Immunoglobulin Allotypes

In the spirit of the times, the clonal selection debate did not sour my relationship with Mel Cohn, with whom it has always been fun to argue about anything. In any event, when Mel decided to leave Stanford in 1962, he "willed" the medical student working in his laboratory to me. John Wunderlich thus joined our group, bringing with him a project focused on producing antisera that would distinguish between antibody molecules produced in different mouse strains and putatively encoded by different alleles in those strains (5).

Ultimately, this project blossomed into a full-scale study of the genetics of the Ig heavy-chain (IgH) chromosome region (6). Long before the structure of the IgH region was defined by molecular methods, studies with anti-isotype and antiallotype antisera showed that IgH isotypes are encoded by a series of closely linked loci and that various mouse strains have distinctive alleles at these loci. The IgH isotypes were defined by other laboratories; we produced many of the antiallotype sera and used these sera in genetic studies (gel immunoprecipitation and radioimmune assay) to demonstrate the close linkage of several of the IgH (isotype) loci. In addition, we defined a series of IgH haplotypes based on the combinations of alleles represented at the IgH constant region loci on the IgH chromosome in each of the standard mouse strains and showed that these were codominantly inherited. Interestingly, the IgH haplotypes defined in this way provided the basis for the Jan Klein and Don Shreffler model for organization of the MHC chromosome region.

We reported our IgH genetic studies at a Cold Spring Harbor Symposium (7) at which Henry Kunkel presented evidence for similar close linkage of the human IgH loci. Together, these studies solidified a paradigm that was extended by the recognition of additional loci and haplotypes and laid the groundwork for the modern understanding of Ig rearrangement, isotype switching, and haplotype (allelic) exclusion during B-cell development.

## Regulation of Memory-B-Cell Expression

LEE: Although I was working actively on allotype genetics, I maintained an independent interest in maternal immunization to fetal antigens (H-2 in particular) and in the effects of such immunization on the developing fetus. Therefore, when maternal antiallotype antibodies were shown to pass to the fetus and to delay the initial appearance of the paternal allotype in allotype heterozygotes, Len suggested that I get this to work with some of our mouse strains. I did, further extending my independent work in the lab.

Ultimately, Len's suggestion led us to the discovery of "chronic" allotype suppression. This occurs when SJL males are mated to immunized BALB/C females producing high-titer antiallotype antibodies reactive with the paternal Igh-1b (IgG2a) allotype. This finding then led to the discovery and characterization of CD8 suppressor T cells that control the expression of IgG2a (Igh-1b) memory B cells without impacting survival of the memory population (8).

While exploring the mechanism(s) underlying allotype suppression, we recognized (as others had before us) that priming with typical protein antigens enabled a strong secondary response to the determinants present on the priming antigen. Such priming also resulted in suppressed responses to new epitopes such as haptens introduced on the priming antigen at the time of the secondary challenge. Because this suppression persists when carrier/hapten-carrier-immunized animals are challenged with the new epitope (hapten) on a different carrier protein, we refer to it as epitope-specific suppression (9).

Although these studies were highly rigorous, they were not met with universal acclaim, perhaps because of the confusion they introduced and perhaps because they occurred just at the dawn of the molecular era in immunology. Nevertheless, the findings are "alive and well" in the vaccine world, where they have been confirmed with a variety of antigens and provide an important caveat when generating vaccine strategies. Similarly, the immunoregulatory-circuits model we had constructed just prior to beginning the epitope-specific work (10), which predicted much of what we found, was not roundly embraced by immunologists, but it too is alive and well, I am told, among today's immune-system model builders.

## THE FACS

LEN: As I became more deeply involved in immunology, I became increasingly aware of the need to characterize and isolate the different kinds of lymphocytes that were beginning to be visualized with fluorescent-labeled antibodies under the microscope and studied functionally by sensitivity to complement-mediated depletion after treatment with antibodies (in conventional antisera). The need for better cell-isolation methods here dovetailed completely with the need for developing a method for positive selection of variants in the somatic-cell genetics projects that I was also engaged in. So I started asking around to see whether anyone had solved this problem.

I soon found out that a group at Los Alamos (led by Mack Fulwyler and Marvin Van Dilla) had developed a machine that could examine and sort large numbers of cell-sized particles on the basis of particle volume. I immediately planned a trip to see whether I could convince them to add a fluorescence-detection system so I could use their machine to measure the amount of fluorescence associated with individual cells and to sort cells according to this measure in addition to volume. They demurred, saying that this "was not part of their mission." [They were funded to build a machine to count and size particles, not cells, obtained from the lungs of mice and rats sent up in balloons to inhale debris generated by atomic-bomb testing (11).] I persisted, and they finally agreed to give me a set of engineering drawings and the permission to use them as the basis of a machine designed to distinguish cells labeled with fluorescent antibodies. Little did I know when I brought these plans back to Stanford that I was starting on a lifework that continues today as a major activity in our laboratory.

Back at Stanford, I took advantage of my close proximity to the instrumentation research laboratory set up by Joshua Lederberg to look for life in outer space (on Mars or on the Moon). I asked the engineer I knew best to look at the plans and estimate the cost to replicate the Los Alamos machine. He came to me a few days later and said, "Okay, I've got good news and I've got bad news. Which do you want to hear first?" I opted for the good news, and he said, "Well, I think the machine can be built here and I've completed a list of parts to be ordered." I asked what it would cost, and he answered, "Something like $14,000." That was a lot for those days, but it could probably be managed. So I asked him, "What's the bad news?" He answered, "The bad news is that I'm leaving Stanford. I've got another position."

I next talked to Josh and the head of the Instrumentation Laboratory. They agreed that despite the loss of this key engineer, they could provide the engineering help I needed for the project. I went to Henry Kaplan, who was head of the Radiology Department and was working on thymic function and development (Irving Weissman worked with him). I told him how I thought a fluorescence-based cell-analysis and -sorting machine could be used to study the thymus and asked him to join me in funding the development of this machine. He agreed. I put up $7,000 from my somatic-cell genetics grant, he put up the remaining $7,000 needed to meet the estimate, and the project got under way.

I didn't do any of the engineering on this project. However, I was deeply involved in the daily development. I was essentially the head of the design team and took responsibility for assuring that the machine would be usable by scientists doing immunological or genetic studies. For example, at one meeting, the engineers told me that the best they could do was to take data from about one million cells in an hour. This was too few to be useful, so I insisted that they either increase the speed by an order of magnitude or close the project down. At first there was some discussion about "repealing the laws of physics," but eventually an engineer came up with a solution and we were off and running again.

I was also responsible for getting new capabilities designed and tested. I loved this role because it encouraged me to think broadly about potential applications for the nascent FACS and to develop collaborations within and outside our laboratory to generate and test these kinds of ideas. In fact, although FACS development has long since ceased to be an activity occurring solely within my purview, I still enjoy the development of new FACS applications and the scientific breakthroughs such development engenders.

Our first cell-sorting paper was published in *Science* in 1969 and was entitled "Cell sorting: automated separation of mammalian [plasma] cells as a function of intracellular fluorescence" (12). The instrument we used for this study had a xenon light source, which we replaced with a laser shortly thereafter.

By 1972, we had developed a much improved instrument and decided to call it the Fluorescence-Activated Cell Sorter (FACS). The engineering team was also much improved because I was able to recruit Richard Sweet, inventor of the ink-jet printer, to head the team. In essence, I pointed out to Dick that the sorting module

in the FACS was based on his invention and asked him to join our group. He responded, "There's nothing I'd like to do more. I'd like to see biological applications of my inventions." And see he did, as he applied himself to the development of several of the core features still with us in the modern FACS instrument.

Dick's initial work generated a paper, published in the *Review of Scientific Instruments*, that was really the first one describing the modern FACS (13). He also joined us as an author of a 1976 *Scientific American* article in which we introduced the FACS and the idea of using this novel instrument to track the expression of genes encoding surface molecules that distinguish various kinds of lymphocytes and other cells (14).

## FACS Goes Commercial

The next major milestone in the development of the FACS was a meeting I had with a vice president of Becton-Dickinson (BD), parent company of the current BD Biosystems (BDB), and with Bernie Shoor, then a local BD representative who ran an engineering group in the Stanford area. Bernie and the vice president came to me because they wanted help with making (conventional) antibodies. I changed the subject and said, "Well if you're interested in making antibodies, then you're interested in immunology. The most exciting thing in immunology right now is our fluorescence-activated cell analysis and sorting [FACS] instrument, which we have been developing for some time and is now working!"

Bernie was interested in the machine but didn't think it was commercially viable. "I think maybe we could sell 10 of these instruments worldwide," he said. I thought 30, or possibly as high as 100 sales were more likely, but neither number seemed high enough to BD to support turning FACS into a commercial machine. BD, in the person of Bernie Shoor, would have walked away from the venture if I hadn't gotten an NIH contract that would let me subcontract the building of two such instruments to Bernie's group and let me collaborate in the effort. It's also true, though, that I might not have gotten the contract if I did not have BD on board to build it.

We eventually built two FACS instruments, one for Stanford and one for the National Cancer Institute (NCI), which put up the money as part of the "war on cancer" (15). Within a short time (as such projects go), we had a commercial instrument to replace "Whizzer," the breadboard model we had been running until then. FACS-1 was later upgraded to FACS-2, which ran for many years both in our laboratory and at the NCI. Ultimately, the NCI instrument wound up in an NIH museum. Our instrument went to the Smithsonian Institute in Washington, DC, and is presently on display at the Walter Reed Army Institute of Pathology. The Walter Reed exhibit includes a tape recording of me describing some of the early work our group did and some of the work done by Bernie Shoor and his group.

After the NIH contract work was completed, BD successfully marketed FACS-2 and we continued our independent development effort. Within the next few years,

we had made some key improvements, including the addition of fluorescence-compensation circuitry to correct for spectral overlap between dyes and four-decade logarithmic amplifiers to allow the full range of FACS data to be displayed on a single data plot. In addition, we introduced the use of computers for data collection and built the first software for FACS data computation and display.

## Monoclonal Antibodies as FACS Reagents

LEE: In 1975, just about the time that Cesar Milstein and George Kohler succeeded in immortalizing spleen cells that produce antibodies by fusing them with a long-established myeloma cell line, Len arranged a sabbatical in Cesar's laboratory at the Medical Research Council (MRC) in Cambridge, England. He chose the MRC to learn the new molecular biology methods (which he did). However, by the time we reached Cambridge in the fall of 1976, the fusion work was in full swing, and Len was quick to realize that the ability to produce monoclonal antibodies to cell-surface determinants would remove what had come to be the most irritating restriction to FACS work at the time.

The conventional antibody reagents that we were using for FACS studies were made primarily in mice or rats and were always in short supply. Furthermore, the specificity was always questionable because the animals were immunized with cell preparations that contained many different potential antigens. Finally, the ability to produce directly conjugated reagents was very limited, making background staining by the second-step reagents a major problem. No wonder then that Len was anxious to tap this new monoclonal reagent resource.

Cesar, on the other hand, had not attended the many meetings we had at which discussions of potential problems with conventional antibody reagents had been narrowed down to the need for groups to exchange staining reagents before the findings could be evaluated. Therefore, Cesar was not highly motivated to have Len delve into the monoclonal technology and urged him instead to pursue the molecular biology studies he had come to do.

The solution to this came when Vernon Oi, then a graduate student in our laboratory at Stanford, came to Cambridge for a prolonged stay. I was working, in principle, at the Babraham laboratories with Arnold Feinstein. However, I had not made much use of the space Arnold gave me because I had to write several chapters for the *Weir Handbook of Experimental Immunology* as well as several papers that had piled up before I left Stanford. Arnold was pleased to let Vernon take my place, and with agreement from Cesar, he outfitted a laboratory in which Vernon (with help from Len and me) could make a set of monoclonal antibody reagents that would detect allotypic determinants on IgG molecules (16).

We brought this technology home at the end of the sabbatical year and, with a FACS available for screening for antibodies to cell surface determinants, began making a series of monoclonal reagents (17) to mouse MHC and other cell-surface molecules (16, 18). Shortly thereafter, we made a unilateral decision to make our monoclonal reagents, and the cell lines that produced them, freely available to the

scientific community. I had the pleasure of announcing this at a large MHC workshop meeting and was pleased when Baruch Benacerraf approached me after the session to compliment our laboratory specifically on this decision. Breaking into the circle surrounding me, he said, "I would like to shake your hand." And he did!

Len also recognized at this time that distributing cell lines that produce important monoclonal reagents would not be sufficient to ensure the availability of these reagents to the overall immunology community. While still in Cambridge, he had phoned Bernie Shoor to suggest that he get BD to set up a commercial mechanism for producing and distributing monoclonal FACS reagents. It took some time for this to occur, but BD ultimately set up a business whose growth and importance to research and medical practice has well validated the original idea.

Interestingly, neither BD nor we thought it necessary or appropriate to patent the monoclonal reagents that the BD monoclonal center was producing, or even to restrict the dissemination of the cell lines that produce these antibodies. Bernie felt, and was proven correct, that people would prefer to buy well-characterized fluorochrome-conjugated reagents rather than produce these reagents themselves. This view is probably more correct now than it was at the time. However, it became untenable as patents for biological material became commonplace and suits for patent infringement began invading the biomedical arena.

Some time later (1982), Vernon Oi and I teamed up with Sherie Morrison (on sabbatical leave with Paul Berg at Stanford at the time and now at UCLA) to make human/mouse chimeric antibodies in which the antibody specificity was encoded by variable-region genes derived from mouse and the heavy-chain constant region was encoded by human IgH genes (19). Because we believed that chimeric antibodies of this type were likely to be useful as functional antibodies and therapeutic reagents, we applied for a patent for this molecular method (issued in 1998). We have been pleased to see the method applied by others, e.g., in the production of chimeric anti-TNF-$\alpha$ used in the treatment of human autoimmune diseases.

## FACS: The First Biotech Instrument?

If the biotechnology (biotech) industry can appropriately be characterized as an industry built around defining, measuring, and making use of gene expression in biology and medicine, then the FACS as we built, described, and used it in the early 1970s readily qualifies as a biotech instrument. In fact, to our knowledge, it is the first such instrument. In addition, Garry Nolan (Medical Microbiology and Immunology, Stanford) points out that FACS should be recognized as a key proteomics instrument, since it has been used in numerous studies to define the functions and demonstrate the interactions of surface and intracellular proteins. Although titles shouldn't really matter in science, it is appropriate to grant the FACS these distinctions. Similarly, it is appropriate to congratulate Bernie Shoor and BD for having had the foresight to build the first biotech company and to lay the foundations for it to grow to its current status as Becton-Dickinson Biosciences (BDB).

Len, of course, has been honored many times for his innovative role in developing the FACS and demonstrating its applications in biology and medicine. Notably, he was cited for this work when elected to the National Academy of Sciences in 1982.

## Breaking the FACS Color Barrier

LEN: At the beginning of the 1980s, we realized that the immunology and other studies that we wanted to do were limited by the number of individual fluorescence measurements (sometimes called parameters) that a single-laser instrument could make on individual cells. There were enough markers known on T cells, for example, to suggest that multiple subsets existed. However, we recognized that using these markers effectively requires their simultaneous measurement on individual cells. Measuring their expression two by two, or even three by three, is not adequate. Information is lost when the measurements are separated and cannot be regained by trying to merge them during analysis. David Parks, then a member and later the leader of our FACS Development Group, solved this problem by extending the FACS-2 to create a dual-laser FACS instrument that would, at a minimum, double the number of markers we could measure on a given cell. In addition, he independently developed single-cell cloning and added this capability to the dual laser instrument (20–22).

This was not the first dual-laser FACS (one had already been created by an instrumentation research group in Germany). However, it was the first dual-laser instrument put into routine use for immunologic studies and hence was the first instrument to demonstrate the effectiveness of using multiparameter FACS methods for distinguishing lymphocyte subsets and for sorting these subsets for functional studies. Many of the key findings made over the years by our group and by other research groups at Stanford were enabled by the development of this dual-laser instrument and its installation in the Stanford Shared FACS Facility, which Len helped to organize some years ago and which David Parks now directs.

We put the dual-laser instrument into routine service in 1983. Roughly 15 years later (1998), we put into operation a hybrid instrument (BD bench, Cytomation electronics) that provides three independent laser illuminations and can simultaneously measure up to 11 distinct fluorescence emissions from individual cells. The number of markers measured with this high-definition (Hi-D) FACS (23–25) instrument, and with our recently purchased BDB Hi-D instruments (FACS DIVA and ARIA), has grown from an initial 8 to the current 11, now the standard for most work in our laboratory. Mario Roederer and his group at the NIH have extended FACS DIVA and located additional fluorescent dyes that can be measured simultaneously to further increase the number of measurements that can be made per cell.

## The Soft Side of FACS

LEE: The original FACS data were collected by photographing histograms traced on an oscilloscope screen. Although these were the early days for using computers

to collect data from laboratory instruments, we once again were able to capitalize on our connection with Josh Lederberg's exobiology engineering group and, with their help, began using the Digital PDP-8 computer to collect FACS data. Wayne Moore, who joined the FACS development group shortly thereafter and has since built or supervised the building of all of our FACS software, moved the FACS data collection and analysis to the PDP-11 platform. On this platform, he developed models for much of the data analysis and display methods that are still in use, including the equal-density (probability) contouring method that is today's standard.

FACS/Desk, which Moore introduced at about the same time that our dual-laser FACS was put into operation (26), was built on a VAX-11/780 platform and offered a nonprocedural (keystroke rather than command line) user interface. This interface, which had windows that opened and asked for user input, foreshadowed what I was later to see in the Apple Macintosh windowing environment.

Sometime around 1980, Len had to raise nearly half a million dollars to buy the VAX computer and build the specialized computer facility necessary to house it, but we have always considered this well worth the trouble. The capabilities that Moore's full FACS/Desk system provided, and still provides, have enabled countless large multiparameter experiments and have provided a permanent, searchable record of all FACS experiments done in the Stanford Shared FACS Facility.

For the past several years, we have been working on a replacement for FACS/Desk. Some time ago, we did the initial designs for a new FACS analysis package. These provided the basis for Mario Roederer's extensions and ultimately for the commercially developed FlowJo package (TreeStar.com), which is widely used today. At present, we are migrating data stored in FACS/Desk to our new FACS DataStore, whose capabilities are much improved over the older system, and have completed an initial version of a searchable Directory Server that can be closely integrated with the new DataStore.

We, in collaboration with Mark Musen, Medical Information Sciences, Stanford, and Stephen Meehan, Meehan Metaspace, are also about to complete the first version of a FACS protocol editor (FacsXpert) that provides an advanced user interface coupled to knowledge-based technology to facilitate design of 12-color FACS staining protocols. FacsXpert is also designed to "painlessly" capture the information (metadata) necessary to annotate data for analysis output (e.g., for axes and table heads) and to facilitate searches with the Directory Server. Ultimately, we hope to make all these capabilities available to the scientific community and to extend the system to take and store data from multiple instruments. This goal, however, may have to wait until we can find a willing and appropriate commercial partner.

Len has been both contributive and supportive in this software development effort. However, in some ways it has been very much "my baby." Although I have written only a small part of the overall system (specifically, the FACS Facility instrument scheduler), I have frequently participated in the design process and have opened relevant collaborations with colleagues in the Stanford Computer Science

and Statistics Departments. In addition, I pioneered the connection of FACS/Desk and FACS analysis output to the SAS Institute JMP statistics package to enable analysis of data from our HIV clinical trials. For this effort, and for the development of the overall FACS/Desk system, we were awarded the Computer World Smithsonian Award in recognition of our visionary use of information technology in the field of medicine.

### A Note about Innovation

Although all the key flow cytometry technologies that we developed were eventually adopted as standards by the commercial and academic flow-cytometry community, we have routinely encountered substantial resistance to the initial spread of these technologies. Biological innovations such as the use of monoclonal antibodies as FACS reagents were readily and rapidly accepted (27). However, technological innovations not part of the biological idiom fared less well, particularly when statistical or mathematical treatments were involved. Hopefully, this will change as these modes of data analysis become more common within the biomedical research community.

## THE FACS IN THE SERVICE OF IMMUNOLOGY (AND VICE VERSA)

LEN: This and the following sections summarize the work we have done over the past 50 years, organized longitudinally by subject area. Presenting the work this way provides a clear view of how our interests in various areas have played out, but it tends to obscure the ways in which the work in each area influenced the development of work in the others. This interplay, generated by the simultaneous pursuit of diverse studies within Lee's and my jointly run group, is one of the key elements in what might be termed the Herzenberg laboratory experience.

Interactions with our contemporaries at other institutions also played a key role in shaping our work. It's hard to convey the fun we all had working individually, but nonetheless as a group, to unfold the immune system and pry its secrets loose. Immunology, particularly the study of cells and cell functions in the immune system, was a small discipline at the time. By and large, those of us working in this area communicated with each other frequently. We made a point of sharing reagents, knowledge, students, and fellows in a way that has become more difficult as we have gotten older and the field has gotten larger. But although our specific interests have diverged and the work of our close circle of collaborators has overlapped less, we remain friends and still love an evening of "talking science" over a good bottle of wine.

Av Mitchison, Gus Nossal, and Olli Makela, as we have indicated, were part of this early immunology group, as were Tomio Tada, Klaus Rajewsky, Richard Gershon, Bill Paul, Max Cooper, Charlie Janeway, Eli Sercarz, Ray Owen, Hugh

McDevitt, Ben Pernis, and Spedding Micklem. Elizabeth Simpson, Irving Weissman, Robert Mishell, Patricia Jones, Harvey Cantor, Ko Okumura, Lee Hood, and Fred Alt formed a younger contingent with whom we also interacted frequently. Of course there were many others, including the students and fellows in our laboratory (whom we have named by reference). But when we think of "the old days," the people we have named here immediately come to mind as the friends and colleagues who contributed most to our development as immunologists and scientists.

## IgH Allelic and Haplotype Exclusion

Our first serious application of FACS in immunology was done using the breadboard machine to characterize, sort, and transfer rabbit B cells according to Ig allotype expression. These early studies confirmed that the Ig-bearing cells in spleen and lymph nodes are precursors of antibody-producing cells, a conclusion that had been based, until this point, on sensible logic and correlations between enrichment of Ig-bearing cells and increases in functional activity in adoptive transfer studies (28). In addition, these studies provided the first indication that the allelic exclusion visible in IgG- and IgA-producing plasma cells in allotype heterozygotes has already occurred in the B cells that give rise to these plasma cells (29, 30).

In subsequent studies conducted with FACS-1 and FACS-2, we used murine IgH allotype markers on IgM, IgD, IgG, and IgA to further characterize the Ig isotype and allotype commitment of naive B cells and their memory ($IgG^+$)-B-cell progeny. These studies, which showed that a B cell and its progeny are committed to producing Ig heavy chains encoded by only one of the two parental IgH chromosomes (haplotypes), led us to propose that allotype exclusion should more appropriately be called haplotype exclusion (6, 7). Thus, this work laid the groundwork for current understanding of IgH rearrangement and isotype-switching mechanisms.

In additional B cell studies, we showed that the IgG isotype expressed on memory cells indicates the commitment of its Ig-producing cell progeny (31) and that specificity of the surface Ig on memory cells indicates the specificity of the adoptive response they will produce (32, 33). This latter work, done by sorting cells that bound keyhole limpet hemocyanin (KLH), also provided direct evidence for the one-cell/one-antibody concept, which was still an issue at the time.

## B Cells Subdivided

LEE: Randy Hardy and Kyoko Hayakawa arrived in our laboratory just about the time that David Parks brought the dual-laser (multiparameter) FACS to a location where it could be used regularly for immunology studies. Several T-cell subsets were already known at this point, and others seemed imminent. B cells, in contrast, were thought to be largely homogenous except for the kinds and amounts of Ig molecules they expressed. Although we were principally interested in doing functional studies with T cells, we decided to exercise the new instrument on something

relatively simple by focusing it on an investigation of B-cell heterogeneity in the spleen. We figured that three months should wrap up this B-cell work, and then we could get on to T cells.

Wrong guess! We immediately uncovered several B-cell subsets (34–36) and found a third (B-1) shortly thereafter (37). In fact, two decades later, we and a host of other laboratories are still working on a definition of the B-cell subsets within the mature B-cell population and of the developmental subdivisions that occur as unrearranged progenitors differentiate to mature B cells in bone marrow. In our most recent work, we have used Hi-D FACS methods to examine these developmental subsets, as have Hardy and Hayakawa, whose pioneering work in their own laboratories some time ago defined the basic stages (so-called Fractions A-F) of B-cell development and identified many of the key genes expressed at these stages (38).

Our studies of B-1 cells, which express low levels of CD5 and have quite different immune response properties, have spawned a great deal of controversy over whether B-1 and B-2 cells derive from the same or different progenitors (39, 40). However, the key evidence still supports placing these cells in separate developmental lineages. In essence, we and others have repeatedly confirmed the results of our original cotransfer studies, which show that progenitors from adult bone marrow give rise to few, if any, B-1 cells in adoptive recipients in which cotransferred progenitors from fetal liver fully repopulate the B-1 compartment (41–45).

We agree that CD5 expression can be induced on bone marrow–derived (B-2) cells under some conditions and that selection impacts B-1 and B-2 development differently. However, these findings are consistent with either a one-lineage or a two-lineage model. In fact, all the data advanced so far in favor of the one-lineage model are also explainable within a two-lineage model. Therefore, until a compelling reason emerges to ignore the cotransfer results, which clearly favor separate lineages for B-1 and B-2, we will stay in the two-lineage camp.

## T Cells Subdivided and a Bit More on B Cells

LEN: The idea that Ig-bearing (B) cells produce antibodies and T cells help them to do so emerged as a paradigm just about the time that FACS-1 was delivered to our laboratory. FACS sorting, transfer, and cell-culture studies with the new machine completed the proof needed for this basic concept and, by introducing adoptive cotransfer methods into the lab, set the stage for much of our memory-B-cell and allotype suppression studies over the next decade. Shortly before we left for sabbatical at Cesar Milstein's laboratory, we completed a collaborative study with Harvey Cantor and Ted Boyse that provided the first evidence distinguishing the helper and suppressor/cytotoxic T-cell subsets from each other on the basis of reactivity with conventional antibodies to Lyt-1 and Lyt-2 (46).

When we returned from sabbatical, the first monoclonal antibodies that we produced to mouse cell-surface antigens contained several that stained T cells (18,

47–50). Studies with these antibodies brought our focus back to the T-cell subsets (51, 52), but (amusingly, from a current perspective) in a way that also wound up creating a mini tempest in a pint-sized teapot. One of the antibodies we produced reacted with a T-cell surface molecule whose physical properties and distribution on T cells corresponded to Lyt-2 (now known as CD8) and a second antibody reacted with a molecule (now known as CD5) whose physical properties were identical to those reported for Lyt-1. However, the expression of this molecule on T cells, as detected by FACS, did not match the expected pattern. Rather than being restricted to a helper-T-cell subset, it was detectable on all T cells (18, 47). [In fact, as later studies showed, it is also expressed on developing thymocytes and is even present on a subset of B cells (37, 50).]

The demonstration that the Lyt-1 (CD5) molecule is expressed at roughly the same level on all T cells caused some consternation. It clearly did not erode the functional distinction between the helper and suppressor/cytotoxic subsets; but, for the moment, it scuttled the idea that helper and suppressor/cytotoxic T cells each express a unique surface antigen. This was resolved (and the mini tempest dissipated) some time later by the isolation of a monoclonal antibody to human cell surface (CD4) and the isolation, by Frank Fitch, of a monoclonal antibody (L3T4) that reacts with the corresponding murine molecule (53).

LEE: From T-cell subsets in mice, we extended our studies to human T cells. Bernie Shoor (still at BD) put us in contact with Robbie Evans at Rockefeller Institute, who was looking for someone to characterize the monoclonal antibodies he had made to human lymphocyte surface antigens. The work with these antibodies revealed the amazing homology, both at the molecular and the distributional level, of the markers defining human and mouse T-cell subsets (54). It also led Len to comment one day that, "really, the best study of man is man," and it refocused much of our laboratory's energy onto human lymphocyte studies.

## T-Cell Subsets in Disease

The loss of CD4 T cells in HIV disease is well known. However, the selectivity of this loss is overrated. Multiparameter FACS analyses of naive T cells in PBMC samples from HIV-infected people at various stages of disease shows that CD8 naive T cells are lost at the same rate as CD4 naive T cells (55). CD4 memory T cells are also lost, although not as quickly as CD4 and CD8 naive T cells. CD8 memory T cells, in contrast, increase in frequency as HIV disease progresses and are only lost at the end stages of the disease (55). Consistent with the idea that the coordinate loss of naive T cells in both subsets reflects the loss of thymic function during HIV progression, we have shown that both subsets of naive T cells are greatly decreased or missing entirely in recovered Hodgkin's patients treated with radiation to the thymic region (56).

In more recent studies on T-cell subsets, we have used 8 to 11 fluorescence colors in Hi-D FACS studies characterizing cytokine production, TCR antigen

("tetramer") binding, glutathione levels, metabolic markers, and other properties of the naive- and memory-T-cell subsets as well as subsets within these subsets (57–59). The introduction of BD's DIVA and ARIA instruments, both capable of 12-color FACS studies, has now enabled these kinds of studies in other laboratories. Mario Roederer, who pioneered much of the Hi-D FACS subset analysis while in our lab, is now doing forefront work in this area in his own laboratory at the NIH (60).

## Somatic Cell Genetics, Modern Style

LEN: I have often pointed out that monoclonal antibodies and the FACS are complementary tools that synergize to enable studies for which neither alone is sufficient (27, 61). For somatic-cell genetics, the joint use of these tools allowed us (and others) to use the cell lines that produce monoclonal antibodies to investigate isotype expression (isotype switch variants) (62) and antigen-combining site variants (affinity variants) (20). In addition, of key importance for gene cloning and expression studies, these complementary tools provide the ability to select cells expressing particular molecules or variants thereof. Thus, among other things, they allowed us to clone the murine CD8 and CD5 genes (63–65) and to explore the gene amplification (double-minute/minichromosome) mechanism (66) that controlled the level of expression of the CD8 gene cloned into a nonlymphoid cell line.

## Fetal Cells in Maternal Circulation

Many years ago, when the FACS was still a "baby," we decided to use it to see if we could detect fetal cells in human maternal circulation as the first step toward developing a noninvasive method for prenatal diagnosis. We were indeed able to detect the fetal cells (67). However, we didn't follow the project much farther, in part because there was reason to suspect that the cells we detected might not have derived from the current fetus. Indeed, current studies show that fetal cells persist for long times in the mother. In addition, these studies surprisingly now indicate that the presence of these cells is closely associated with the development of maternal autoimmune disease and that maternal cells are found in small numbers in multiple tissues of the newborn (68, 69).

## HIV, NF–$\kappa$b, and Redox

LEE: Living in the San Francisco area in the early days of the AIDS epidemic was like living in a war zone, where the dead were counted weekly, and the casualty lists frequently included friends or friends of friends. In this climate, it was impossible to go to work in the laboratory without wondering whether something you were doing could be of help. Therefore, we took an interest in a letter sent by Wolf Droge (Heidelberg, Germany) reporting that glutathione (GSH), a multifunctional cysteine-containing tripeptide, is depleted in HIV disease and suggesting, on the basis of his in vitro work charting nutritional requirements for T-cell function, that

the GSH depletion results in oxidative stress that may decrease the function of the dwindling number of T cells in HIV patients (70). He also showed that GSH levels were low at the later stages of HIV disease (71).

Droge recommended treating HIV-infected people with N-acetylcysteine (NAC), a well-known nontoxic cysteine prodrug used clinically to prevent hepatotoxicity by providing the cysteine necessary to replenish GSH depleted by acetaminophen overdose. In addition, he reported that his neighbor's HIV-infected son was greatly helped by oral NAC administration. This anecdotal finding was not highly convincing. However, we respected Droge's logic and began seriously exploring the idea of NAC treatment for HIV disease.

In the laboratory, we began what appeared to be an independent HIV project in which we made a construct that put the $\beta$-galactosidase (LacZ) gene under the control of the HIV-LTR and developed FACS methods for measuring the activity of this reporter gene. However, two projects merged when, after hearing Tony Fauci speak about TNF-$\alpha$ triggering of HIV replication, we arranged to test whether NAC would inhibit triggering of the HIV-LTR by TNF (72). The results of these studies provided the first evidence demonstrating that intracellular GSH levels regulate NF-$\kappa$b induction and HIV-LTR activity in cell lines (73, 74).

Importantly for T-cell function, we also showed that intracellular GSH regulates TCR-stimulated calcium flux in cell lines and in primary human T cells in vitro. The Heidelberg group extended this finding by showing, in their placebo-controlled studies with HIV patients, that NAC treatment improves the in vitro function of T cells taken from these patients (75). In the placebo-controlled trial that we conducted sometime before, we demonstrated that NAC treatment replenishes GSH in HIV-infected people (76). We did not detect any impact of the NAC treatment on HIV viral load. However, we found a marked correlation between NAC treatment and improved survival in the open-label portion of this trial, which was conducted prior to introduction of the more effective, current antiviral therapies (76, 77). Collectively, these findings are consistent with NAC being a useful adjunct therapy in HIV disease, and they support Droge's initial suggestion that GSH replenishment may be most important for maintaining the overall health and defense against opportunistic infection in the HIV patient.

As befits a primarily basic science laboratory, the redox studies we were doing were initially relevant to HIV disease but soon engendered a broader interest in the mechanisms through which intracellular GSH levels influence signal transduction and other physiologic properties of cells. To approach these questions more effectively, we established FACS assays for intracellular GSH levels and, more recently, for surface thiols (57), intracellular thioredoxin, and intracellular protein glutathionylation in primary lymphocytes (78). In addition, we began examining other consequences of GSH depletion (79, 80). This burgeoning project continues unabated in our laboratory and now involves studies with samples from metabolic disease, cystic fibrosis, CLL, HIV, and other types of patients.

At the time we were working with HIV-infected subjects, we found that thioredoxin (Trx), a key intracellular redox molecule, is released into circulation and

blocks neutrophil chemotaxis when present at high levels in blood. Consistent with this finding, we found that among HIV-infected subjects with T cell counts below 200/$\mu$l blood, those with elevated Trx levels died within 15 months, even though they had no signs of ill health at the time of assay. This led us to propose that Trx interference with innate responses may render immune-compromised HIV-infected patients more susceptible to progress to the kinds of opportunistic infections that led to death (81). Interestingly, in vitro studies indicate that NAC treatment decreases thioredoxin release (79).

### Glutathione/Cysteine-Deficiency Disease

Together with a broad series of collaborators with expertise in various aspects of GSH deficiency, we recently completed a literature survey of more than 50 placebo-controlled trials in which beneficial effects were demonstrated for NAC treatment in diseases ranging from chronic bronchitis to diabetes. Collectively, these studies indicate that GSH/cysteine deficiency commonly accompanies a wide variety of clinically important diseases and conditions and that treatment of this deficiency may significantly improve health. Thus, we hope this work will call physicians' attention to this problem and the ways that it can be minimized.

## FAMILY, FRIENDS, AND POLITICS

LEN AND LEE: We have not spoken very much here about our family, although all who know us know that the dividing line between work and family is virtually indistinguishable in our lives. Our students, fellows, and laboratory personnel have in many different ways contributed to the upbringing of our children. Berri, our eldest, now runs the Bicycle Trip (http://www.bicycletrip.com), a bicycle shop in Santa Cruz, California. Janet, or Jana as she is now called, is a singer/songwriter who has just released a CD through Motema Music (http://www.motema.com), a small but up-and-coming record label for which she is the CEO. Rick has become a serious salesman and and a fine potter; and Michael, our Down's Syndrome son, is living nearby and working one afternoon at the lab and the rest of the time at a local workshop. We are pleased that many of our friends and previous coworkers have established independent relationships with our children. We are also pleased that the entire family still puts in political time trying to make the world a better place to live in.

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