FOSTERING COMMUNICATION AND COLLABORATION

The NIH CATALYST

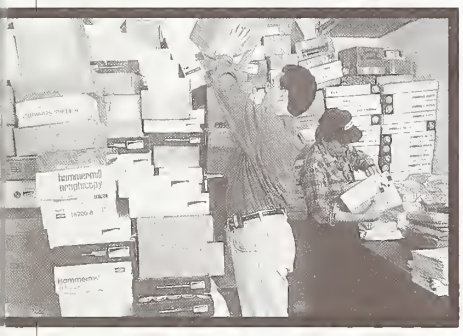
A PUBLICATION FOR NIH INTRAMURAL SCIENTISTS

NATIONAL INSTITUTES OF HEALTH ■ OFFICE OF THE DIRECTOR ■ JULY 1994

THE OTHER NIH: PART II THE NIH GRANT PROCESS AND HOW TO WRITE A SUCCESSFUL GRANT

by Seema Kumar

In the extramural world of soft-money science — a place where getting a grant may mean the difference between a bright scientific future and no future at all — knowing how to get grants is an important part of being a well-rounded scientist, says Jerome Green, Director of the Division of Research Grants



Chris Greggory (left) and Andy Webrle stack some of the more than 10,000 applications NIH receives during each of its three funding cycles.

(DRG). Measured by this criterion, say Green and other extramural scientists, intramural scientists are still a bit rough around the edges.

"Intramural scientists are very good at what they do, but most of them, even senior people, do not know how the grants process in the extramural program works," says Green. What's more, says Judith Greenberg, Program Director of the NIGMS Genetics Program, "many senior intramural scientists are ... not in a good position to advise their outgoing fellows or postdocs, either."

Intramural scientists' lack of grant-

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STATUS OF INTRAMURAL MINORITY SCIENTISTS FINAL REPORT OF THE COMMITTEE

EXECUTIVE SUMMARY

Background and Goals

At the request of the Director of NIH, the NIH Deputy Director for Intramural Research appointed a committee of intramural scientists to examine the status of underrepresented minorities in NIH's Intramural Research Program (IRP-NIH). The committee was to provide a comprehensive view of the issues involved in the recruitment and retention of minorities. The committee included tenured and nontenured scientists and administrative professionals from NIH's Offices of Education and Equal Opportunity and Institute, Center, and Division (ICD) Equal Employment Opportunity (EEO) Officers. The charge to the committee was to document the numbers and percentages of underrepresented minorities among the ranks of tenured and nontenured scientists, to investigate the reasons for this underrepresentation, and to suggest solutions to the problem. The committee was asked to examine the status of minorities in the staff fellowship program, in other programs that train or employ nontenured scientists, and among the ranks of tenured scientists.

THE COMMITTEE WAS TO DOCUMENT THE NUMBERS AND PERCENTAGES OF UNDERREPRESENTED MINORITIES AMONG TENURED AND NONTENURED SCIENTISTS, TO INVESTIGATE THE REASONS FOR THIS UNDERREPRESENTATION, AND TO SUGGEST SOLUTIONS TO THE PROBLEM.

The committee used databases about tenured and nontenured scientists (as of Oct. 1, 1992) that were constructed from information supplied by ICDs of NIH, the Division of Personnel Management, and the Division of Commissioned Personnel. The racial and ethnic status of each scientist was obtained and self-verified by the scientists via a race and national origin form. The data that were used in evaluating recruitment, training, and tenure programs and for conversion to tenure were obtained from the NIH Office of Education.

Tenured and nontenured scientists from underrepresented minority groups (as defined by the American Association of Medical Colleges) were contacted and asked to complete surveys and participate in interviews. A control group of nonminority and nontenured scientists also participated in the study. Results of both the interviews and surveys are summarized here. In addition to quantifying the representation of minority scientists, the committee gathered information on the experiences and perceptions of minorities in the IRP-NIH.

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Increasing Diversity In The NIH Scientific Staff

Michael Gottesman

One of the great pleasures of a scientific career is interacting with creative and productive scientists from many different countries, cultures, and ethnic backgrounds. We take for granted that scientists who are not open to new ideas, who are intellectually biased, and who cannot accept change are doomed to failure in their pursuit of knowledge. Yet a recent report on the role of underrepresented minority scientists at NIH (summarized in this issue of *The Catalyst*) and concerns raised by an advisory group of women scientists suggest that NIH has not done a good job of ensuring equal opportunity for all scientists to work in the intramural program, as reflected in the lack of diversity of its scientific staff and in discrepancies in pay. What can we do to improve the situation?

The major concerns, among many, raised by the *Report on Underrepresented Scientists at the NIH* is that NIH lags behind the average for medical schools with respect to minority representation in tenured positions (2.18% African American, Hispanic, and Native American vs. 3.37% for medical schools on average) and that minority scientists do not feel fully accepted into the "culture" of NIH. Although explanations based on historical hiring patterns, appropriate comparison populations, and the small "pool" size of minority candidates with Ph.D.s in the biological sciences have been proferred, the fact is that we need to do much more to improve minority representation in tenure-track and tenured positions on the NIH campus. Although our goal is to give every qualified minority candidate the opportunity to work at NIH, one widespread perception is that NIH is not willing to open its doors to minority scientists, and that once here, minority scientists are excluded from full participation in the culture of science.

We need to do much more to improve minority representation in tenure-track and tenured positions on the NIH campus.

How do we go about improving minority representation on the campus? One approach is for each of us to make an effort to identify minority colleagues at all levels of seniority who might be interested in doing research here. This involves more than sending out general letters of invitation and expressions of good intentions; we must search out and encourage candidates to apply to NIH. The numerous new tenure-track positions, which we hope will become available as soon as the hiring freeze is lifted, should encourage these applications. Senior-level hiring is also possible; nothing will convince a prospective minority job candidate that we are serious about hiring minorities more than actually improving minority representation on campus.

Where do we find underrepresented minority scientists? A major effort to interest such people in scientific careers is already under way at NIH; our 1992 summer student program minority population was 31.82% for high-school students, 21.84% undergraduate students, and 24.81% medical students. However, summer jobs do not translate immediately into M.D.s and Ph.D.s pursuing research careers; witness the fact that only 3.9% of our postdoctoral fellows belong to groups traditionally underrepresented in the sciences. The reality is that very few minority Ph.D.s are being trained in the United States, resulting in a small pool of candidates, and we need to look to the larger pool of M.D.s who might be interested in research as a source for our next generation of scientists. NIH has done this in the past; witness the large number of M.D.s attracted to NIH during the Vietnam War who now occupy leadership positions in our Laboratories and Branches. We need to find individuals with an interest in research careers and encourage them to come to NIH for appropriate training.

One program with the goal of encouraging minorities to come to NIH is the recently launched Clinical Research Loan Repayment Program (CR-LRP). Modeled on the successful AIDS Research Loan Repayment Program, this program seeks physicians from disadvantaged backgrounds (for our purposes, underrepresented minorities, women, and disabled people may qualify as disadvantaged) whose medical school loans will be repaid over a period of several years while they receive training in clinical research at NIH. Such a program helps us solve several problems: it improves representation of minorities, women, and the disabled at NIH, it creates a talented cadre of clinical researchers at a time when clinical research is under siege, and it provides minority physicians to help us attract minority patients to the Clinical Center's research protocols. The program will have only four participants this year, but we hope funding next year will allow us to support up to 20 new clinical researchers.

Some of you may be aware of analyses by women scientists showing pay inequities at NIH and other problems related to underrepresentation of women in the most prestigious and highest-paying jobs at NIH. I have been working with the Women Scientist Advisors and the Scientific Directors of each of the Institutes to identify all inequities and to try to correct these as they are identified. We do have significant problems in this area, and we will be working to find ways to correct salary discrepancies and to improve representation of women among Laboratory and Branch Chiefs and other high-level positions.

Dr. Varmus, the Institute Directors, the Scientific Directors, and I are committed to improving the representation and career opportunities for minority and women scientists at NIH. We need your ideas and help to improve the current situation. Much more than cosmetic changes will be needed; we must institute substantive changes in the way we recruit scientists, mentor scientists of all colors and cultures when they arrive at NIH, and encourage people to stay once they are successful. I will be working with the Scientific Directors to develop new ways to deal with this issue, and I hope to hear from you with names of candidates for positions at NIH and with ideas for attracting minority candidates.

Michael Gottesman
Acting Deputy Director for
Intramural Research

FAX-BACK FEEDBACK

Below is a sample of the FAX-BACK comments we received for each topic raised in the May issue.

Your suggestions on the External Advisory Committee's report.
"It must be implemented and not shelved like all the previous reports. Has any Task Force report ever been implemented?" — *Anonymous*

"The External Advisory Committee Report places increased emphasis and importance on the review of the intramural research program by the Boards of Scientific Counselors. These reviews will therefore be critical to the career and research advancement of the intramural scientists. However, the intramural scientists have been provided with very little in the way of protection against unjust, incompetent or otherwise faulty reviews: the Report allows the scientist to make a written reply to the review but does not guarantee that any remedial action will by taken. It is therefore very important for the Administration and the intramural scientists to concern themselves with this problem and to see to that a reasonable and effective redress mechanism be incorporated into the review process." — *Judah L. Rosner, NIDDK*

"Increased use of intramural scientists on DRG study sections would increase their awareness and interaction with the extramural community." — *Anonymous*

Your opinions about intramural scientists being ill-prepared for an extramural life and suggestions to remedy it.
"We are terrified of going into research on the outside. If our grants get rejected as frequently as our papers — we will be in BIG TROUBLE, especially now!" — *Anonymous*

"It varies from lab to lab depending on the Chief. Some are eminently prepared to compete; others are in the dark." — *Anonymous*

"If extramural scientists are surmising that intramural scientists are ill-prepared for research life in the outside world, I would guess that they are in error. Perhaps extramural scientists should spend a week in our intramural labs and find out what an intramural scientist's life is really like. Yes, there are differences in either direction you go, but demeaning intramural scientists is very inappropriate. Intramural scientists could enhance their knowledge about the NIH extramural grant process by spending a week or more in the Grants Management Branch of the ICD in which they work. It could also be a great learning experience for Health Scientist Administrators (usually Ph.D.s) to spend time doing the same in the field of their training in an intramural research lab." — *M.G. Marques, NINDS*

"I have been trying to get information on extramural grant writing for 18 months. I have been at NIH for three years and will be required to begin writing for extramural grants July 1 when I begin an academic career in Chicago. I disagree that intramural scientists are ill-prepared for research life in the outside world, but I strongly believe a program is called for that would prepare us for extramural grant writing. I would like to improve my knowledge about the NIH extramural grant process since I am embarrassingly unprepared for this." — *A.K. Pajeau, NINDS*

"As an Extramural HSA I enjoy reading about the intramural programs and scientists in *The NIH Catalyst*. We have so little communication exchange with them. Part 1 of the article on "The Other NIH" by Seema Kumar is on-target relative to the knowledge deficit of intramural scientists on extramural programs and mirrors some of my experiences with departing intramural scientists...who 'discover us' at the last hour. Not knowing the substance of the next two sections, I would hope that Ms. Kumar will include information for the intramural scientist about the existence of research training and career development programs which sometimes are overlooked but go hand-in-hand with the grants programs." — *F. Harding, NHLBI*

Editor's Note: *Seema Kumar will discuss career development and mentorship in Part III of her article on The Other NIH in the next issue.*

Are intramural scientists ill-prepared for the outside world?
"Absolutely not! Especially in terms of research. We also have grant-writing seminars which should be of help." — *Anonymous*

On techniques you would like to see covered in our Hot Method Clinic and your suggestions on in-situ PCR.
"Atomic Force Microscopy (uses of)." — *Anonymous*

"I have used in situ RT-PCR amplification and labeled-probe hybridization to detect several RNA viruses in paraffin-embedded brain tissue. I use a single primer pair to amplify a 300-800 bp sequence, which is then detected with a digoxygenin-labeled cDNA probe, antidigoxygenin antibody-paroxidase conjugate, and diaminodenzadine. Morphology is preserved, background is minimal, and controls remain unstained." — *Stuart H. Isaacson, NINDS*

Your experiences with and suggestions on mentoring and educating young scientists.
"Male M.D.s get substantial mentoring; Ph.D.s less — and women virtually none. To reverse the situation, have each section report on their efforts in writing each year on mentoring activities with a special section devoted to mentoring of women." — *Anonymous*

"I think special attention should be focused on women and minorities." — *Anonymous*

"It should be part of the performance plans! Don't give all the information to SD's and lab chiefs — the real mentoring may be lower down." — *Anonymous* ■

LETTER TO THE EDITOR

Please notify the readership that Dr. William A. Krivoy died in Richardson, Texas, on October 24, 1993. Dr. Krivoy was a well-known electrophysiologist and neurobiologist at Baylor College of Medicine in Houston, and at NIDA's Addiction Research Center. Dr. Krivory was among the first to recognize the importance of neuropeptides as modulators and transmitters in the central nervous system.

Dr. Krivoy worked at NIDA's Addiction Research Center in Lexington, KY, until 1983, when he suffered a massive stroke. Since then, he has convalesced in Texas. Dr. Krivoy is survived by his mother and a brother.

Sincerely yours,
Wallace Pickworth, Ph.D.

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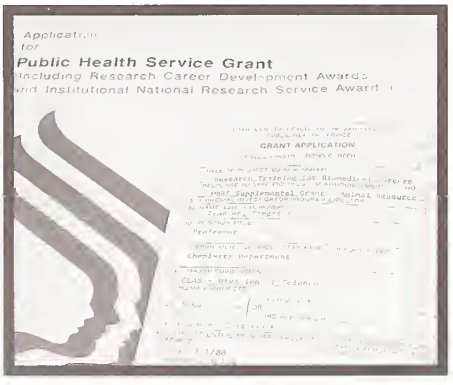
continued from page 1.

writing savvy is understandable — they are, by design, freed of this burden so that they can devote their time and energies to conducting innovative, high-risk research — but it is not excusable. Greenberg says that when they finally leave NIH, intramural researchers and their students may find themselves at a disadvantage if they don't know the workings of the primary source of support for biomedical research in the outside world.

Some intramural scientists acknowledge their ignorance about NIH grant mechanisms and the ins and outs of grant writing. "We are terrified of going into research on the outside," says one anonymous intramural researcher in a FAX-BACK response to part I of this article. "If our grants get rejected as frequently as our papers, we will be in big trouble, especially now."

Typically, this realization hits intramural scientists only when it's time to leave NIH, say extramural NIHers. "Departing intramural scientists often discover us at the last hour," says Fann Harding of NHLBI in her FAX-BACK response to part I of this story. And it comes as no surprise to her and other extramural NIHers that when NIGMS holds its annual course on "How to Apply for a Grant," the course is filled to capacity.


Application for
Public Health Service Grant
(including Research Career Development Awards
and Institutional National Research Service Award)
GRANT APPLICATION

To apply for an NIH grant, use the PHS 398 kit, available at your institution's or NIH's Grants Information office (Ph: 594-7248)

The course, which NIGMS started in 1978 to prepare its departing pharmacology research associate training fellows for grant writing, "was so popular that NIGMS opened it up to the entire intramural community," says Greenberg, who coordinated the course in the 1980s.

The course now serves an important function at NIH, say Green and Greenberg: It provides a training similar to that offered by universities and private companies to train postdocs and others in grant writing. This year, to accommodate more participants, the seminar will be held in Masur Auditorium, and the date has been shifted from spring to Oct. 27, when the information will be more timely for scientists hoping to leave NIH with a grant in hand come next spring.

The important message, says Joan McGowan, Chief of the NIAMS Bone Biology and Bone Research Branch, is that "grant writing is not some arcane field that is difficult to learn. Intramural scientists can easily master the mechanics of grant writing, and this course provides them that opportunity." Intramural scientists may not become experts on grant-writing overnight, but "they will know who to call, where to go and what to do, after this course," says Paul Wolfe, a Program Administrator at the NIGMS Genetics Program and coordinator of this year's course.

In part II of our story on extramural NIH, *The NIH Catalyst* describes key elements of this popular course on NIH grant-writing and peer-review mechanisms. We interviewed, among others, the dean of grant writing, DRG's Green, and Greenberg, intramural-investigator-turned-extramural-administrator.

How to Apply for an NIH Grant

First the basics: to apply for an NIH grant, you must use the PHS 398 kit, which you can get from your institution's grant office or from DRG (Grants Information Office, phone: 594-7248). Applications for new grants are due at DRG on Feb. 1, June 1, or Oct. 1, depending on which of the three annual funding cycles you apply in. If your application has not been funded, you can revise and resubmit the application in the next cycle. "About a third of the 40,000 applications DRG receives each year are revisions of previous applications," says Green. Each revision is reevaluated for scientific and technical merit and for responsiveness to the previous review.

The NIH Peer-Review Process

The heart of the process for selecting among the almost 40,000 applications for NIH funding is the peer-review system, which has evolved over 40 years and is one of the most rigorous and systematic in the world. "Peer review ensures that the applications receive an objective evaluation and helps decide which among the many excellent proposals are the most meritorious and promising," says Green. Extramural peer review is entirely different from the intramural review conducted retrospectively by a Board of Scientific Counselors (BSC), says Green.

Peer review, extramural style, is prospective, says Green. That is, a project is reviewed on the merit of what is proposed for future work. "When we review a grant application, we look at what a scientist plans to do, what his or her objectives, hypotheses, and methods are, what the investigator's experience with an area of research is, and how much money and time the investigator proposes to spend," says Green.

"The extramural peer review system may seem like a bunch of hoops that administrative people have put up for scientists to jump through, but it is in fact a very science-driven process," says McGowan. She stresses that for any project, "good science comes first, and intramural scientists need no lessons in that respect. What they need to learn are the procedural aspects of grant writing."

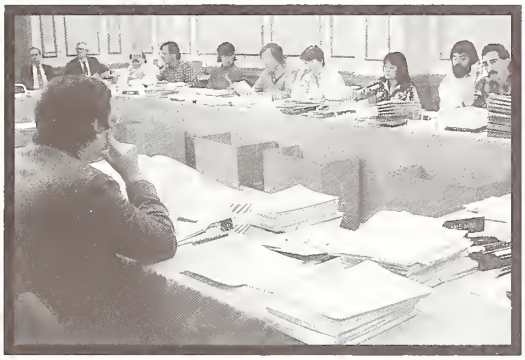
The bottom line, says Green, is that intramural and extramural review differ considerably and, therefore, "it behooves intramural scientists who are planning careers outside to get to know about the NIH extramural review system and to know what their prospects are."

Green says that the potential for making mistakes on grant applications is high, especially if you don't know how the review system works: "You may think, 'My budget may be cut next year, so I'll ask for much more than I need,' and that is bad," says Green. "A panel will look at it and say that this investigator has no realistic idea of how much research costs and that this is a ridiculous request and, so, reject it." On the other hand, says Green, "if you request an amount of money that is grossly insufficient, the panel is likely to reach the same conclusion." Greenberg says the importance of the grant seminar is that it teaches people these subtleties. "It teaches you not only how to apply for a grant, but also the process by which applications are referred to institutes and study sections,

how they are reviewed, what reviewers look for in an application, and how funding decisions are made," says Greenberg.

What Happens After You Apply:

The NIH peer-review process begins at DRG, the central receipt point for all grant applications. DRG processes the applications and assigns each one to a study section and to the most relevant of NIH's 21 funding institutes. Each application is given a unique number, and most are handed over for two levels of review.



Applications undergo a first level of review by one of 100 study sections, each comprising an average of 18 experts from around the country.

"The first round of review is for scientific merit, and 80% of these reviews are done by one of DRG's 100 study sections, each comprising an average of 18 experts from around the country who are appointed for 4 years," says Green. These experts are identified and nominated based on their experience and expertise in a certain area by the Scientific Review Administrator (SRA), an NIH employee who coordinates the study section.

The study sections meet typically for 2 to 3 days, three times a year — once during each funding cycle — and review 75 to 100 applications at each meeting. At these meetings, members discuss each application individually and judge it by the following criteria:

- scientific significance and originality,
- adequacy of methodology,
- qualifications and experience of the principal investigator and staff,
- reasonable availability of resources, and
- reasonableness of the proposed budget and time schedule.

In addition, other criteria, such as provisions for adequate protection of human and animal subjects, and of the environment, come into play. At the end of the discussion on each application, study section members give a priority score for the application if they think it is worth further consideration. Approximately 85% of applications are scored or graded at the study section level, whereas about 15% are not recommended for further consideration.

Every application that is recommended receives a rating, ranging from 1.0 for the most enthusiastic to 5.0 for the least enthusiastic response from the members of the panel. The average of the members' individual ratings is multiplied by 100 to obtain a three-digit priority score, and these scores, are ranked on a percentile basis within each study section. "These scores are important guides to Councils and Institutes to make funding decisions on the grants," says Green.

The SRA then prepares a summary statement for each application, giving a short description of the project, a critique of its strengths and weaknesses, and, for each application that is recommended, the priority score it received, its percentile ranking, and a recommended budget and duration for the project. The grant applications that are recommended for further consideration and their summary statements are forwarded to the appropriate Institute, where they undergo a second level of review by Institute-wide National Advisory Councils (called Councils) or Boards.

Applications undergo a second level of review by Institute-wide National Advisory Councils or Boards.

"Council reviews not only the scientific merit of the project but also how it meets other criteria, such as programmatic needs, portfolio balance, and availability of funds," says Green. On the basis of the study section's scores, the reviewers' comments, the summary statement, Council's recommendations, and the Institute's programmatic considerations and availability of funds, decisions are made regarding the funding. Each Institute has a team of Health Scientist Administrators who help the Institute Director make final decisions about which applications will get funded. "In making these decisions, we don't have to take reviews in perfect numerical-priority-score order," says Greenberg. "We take into account various things such as whether an area is underrepresented."

At the end of this process, "only 20 to 25% of all applications get funded, and the competition is very keen," says Green.

The Recipe for a Successful Grant Application

Now comes the important part: What distinguishes the 20 to 25% of the applications that get funded from those that don't? The answers to this question, says Green, are the ingredients that make for successful grant writing.

"First, start with a good scientific idea," says McGowan. "A good project is, first and foremost, about creative, innovative, and sophisticated science; it is the most important ingredient." That said, "There is more to applying for a grant than being a brilliant scientist," says Green. "You have to know how to present yourself in a written application within certain constraints: you can't have 90 pages describing your proposed work; you are limited to 25 pages."

Reviewers are also interested in investigators' past productivity, says Green. "They want to know: Are you publishing? Are you publishing in good journals? Are you publishing in closely related areas of science?" But the majority of the reviewers' verdict is going to depend on the project, says Green, "which is why we will sometimes get an application from a Nobel Prize winner and it will not do well. The individual may have a fantastic track record, but the project description may be poor."

Green's advice to potential grant applicants is to think the project through well, follow instructions, observe the rules of good writing, and check and double-check for any errors. He also recommends that applicants call DRG or ask

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continued from page 5.

to see a videotape or check out brochures from the DRG Grants Information Office. "We have lots of good material available for the asking," says Samuel Joseloff, who heads this office. Published below are some additional tips that come straight from DRG and their reviewers.

Filling Out the PHS 398 Form.

- Plan your project carefully. Use the same care developing an idea as you would for a research publication.
- Think about your audience: the busy, overworked people reading the application. Clarity, brevity, and economy, getting to the heart of what you want to express, are the key. "Most of the study section members are on the faculty of a university or an institute, and they have their own jobs. They read grant applications at home and in the evenings or on weekends. You want to make it very easy and clear for them," says Green.
- Don't assume reviewers know what you mean. Spell it out. Competition has never been stiffer, and you may have to pay a heavy price for any doubt that you leave in the reviewer's mind.
- Don't leave out potential pitfalls and alternate approaches. The reviewers will think you never even considered them.
- Set up informal pre-reviews of your own: ask some unbiased colleagues, preferably successful grantees, for feedback at every step of the process. Get an editor and allow yourself enough time to incorporate suggestions.

Doing Your Homework

- Adhere strictly to the rules on the number of pages, citations, type size, appendices, reference letters, and overlapping support. Be sure to make provisions for the protection of human and animal subjects.
- Check and double-check everything. Proofread and let someone else do it, too. Check figures on the budget page. If you have any questions about filling out your application, call the Grants Information Office at 594-7248.
- Take extra care preparing a realistic and adequate budget. A poorly conceived budget makes people wonder whether you can manage a project.

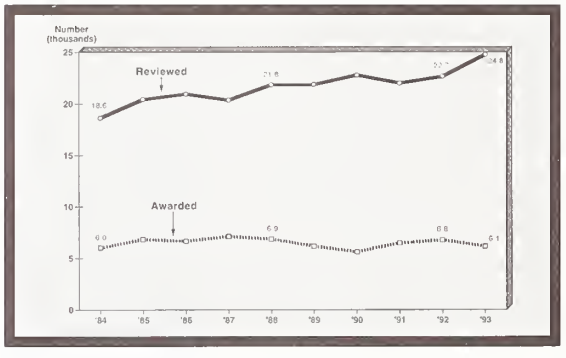
Most Common Reasons for Applications not Being Funded

DRG experts also pinpoint the most common reasons for applications not being funded.

- Not original or significant; it must be good science. "The whole application has to be imaginative, good science and realistic, sophisticated, and mature," says Green.
- Too diffuse, superficial, or unfocused: "Give reviewers enough information to conclude that you are knowledgeable and experienced, and that you are not going after new information with a butterfly net," says Green. State a well-formulated hypothesis.
- Vagueness on where the project is going in the future.
- Questionable reasoning in experimental approach.
- No acceptable scientific rationale.
- Not enough preliminary data to justify the project.
- Proposes an unrealistically large amount of work.
- Too uncritical in approach.

What Happens Next

Let's say you have followed all this advice and have sent a grant application to NIH. What happens next and how soon do you know how you have fared? Six to eight weeks after you apply for a grant at NIH, DRG sends you an acknowledgment and tells you to which institute and study section your application has been assigned. Your contact point, if you have any questions, then becomes the SRA of the study section. During this period, your application is going through the first level of review. A few months later, you will receive a so-called pink sheet — a summary statement with a description of your project, a critique of the project's strengths and weaknesses, and, if it has been recommended, its priority score, percentile ranking, and the recommended budget and time limit. At this point, your application, if it has been recommended, has been forwarded to the appropriate institute, where it undergoes a second level of review at Council.



Because the number of applications NIH reviews has increased over the past decade and the number of awards it has made has not, competition is stiff.

Once the funding decision has been made, you receive word about whether your grant was funded. Program staff members notify grantees about the dollar amounts, which are often different (and usually lower) than the requested amount, and work with the Grants Management Office to send out official notices — memos that initiate payments on grants — to successful applicants.

Throughout this process, NIH administrators try to remain available. "We work with investigators before, during, and after the application process and answer their questions on what NIH or our institute is looking for in the application and discuss the merits of their ideas and the type of grant they should apply for," says McGowan. Program directors also help scientists focus on their scientific areas and guide them through the application process. "For some investigators, the grant-writing process is a scary, nebulous process. What we are here to do, as part of our jobs, is to help them through that process as best as we can, given the resources we have and the time we have," says Dennis Mangan, Director of the Periodontal Diseases Program at NIDR.

But, says Green, "there are no special breaks for an intramural investigator" applying for a grant to work extramurally. Greenberg observes that it wouldn't hurt intramural scientists to get to know some their extramural colleagues before leaving NIH to establish a contact point.

"We have great respect for what intramural scientists do and would love to work with young and senior investigators and help them with any questions they may have about the process of grant writing," says McGowan.

For more information on any aspect of NIH grant writing and review, call the DRG Grants Information Office at 594-7248... *and good luck.* ■

(Look for part III of this story, featuring a discussion on mentoring and career development for young scientists, in the next issue of The NIH Catalyst.*)*

A SUMMER STUDENT'S EXPERIENCE

IN SEARCH OF EPIPHANY

by Stephen Shih, Summer Student, Laboratory of Pathology, DCBDC, NCI

Stephen Shih, a summer student in the Laboratory of Pathology, NCI, wrote this essay as part of his application for his summer position. Ms. Mildred Steinberg, chief program officer for Lance Liotta's pathology lab, was impressed with the essay and passed it along to The NIH Catalyst. *We were as impressed as Ms. Steinberg and think the piece typifies the creativity and liveliness of NIH's summer students, while presenting some insightful observations on life at a critical stage in professional development. We have asked Mr. Shih to write again for* The Catalyst *at the end of the summer, before he returns for his sophomore year at Harvard.*

THE GOALS AND AIMS OF ONE (1) STEPHEN Y. SHIH

Okay, so Mildred Steinberg (who, incidentally, was extraordinarily helpful in my search for summer opportunities — many thanks if you're reading this) says something like this to me over the phone: "Before we start the paperwork to bring you here, we'll need a few things from you: a school transcript, a letter on your school's stationery attesting that you're a student in good standing, and a statement from you concerning your goals in life and for this summer in particular..."

A Statement of Your Goals in Life — a pretty whopping big order. This is the type of topic people wax philosophical on, writing plays, composing epic poems, even choreographing pieces in interpretive dance to chronicle one lost soul's search for purpose in being.

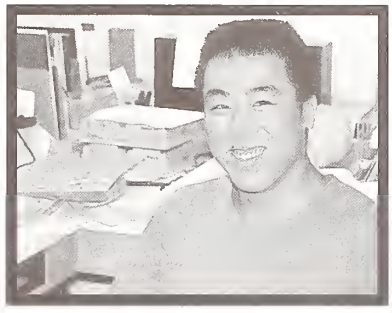
"Uh, so what format do you want this statement in?" I ask.

"Just a page or so, written," she replies.

Oh.

So here I am with the simple request to crystallize my purpose in life and put it on paper. In ink. Like setting it in concrete. At least with those other options (*see*: interpretive dance), there's room to equivocate. "When I grow up I want to be a _____." Find the word to fill in the blank and you've got your whole life figured out — no ifs, ands, ors, or buts. Hell, last month, I had to decide what my major will be, and I'm still not sure I made the right choice.

Ever hear people who are famous in their fields describing how they got their starts? Almost invariably, they have a story about an experience leading to some huge personal revelation: "...and it was at that moment that I knew I was destined to become anactor/astronaut/molecular oncologist!" Well, I'm 19, and I've yet to have that moment of epiphany.



Stephen Shih

I've spent a lot of time wondering where my life is going. In the past, school hasn't helped much. In high school, I always got straight A's, so report cards were never much help in analytically determining my particular aptitudes. Being unsure of where my interests lie left me in sort of an occupational limbo. By turns, I've wanted to be, among other things, a pilot, a writer, and a smoke-jumper. Smoke-jumpers, by the way, are people who parachute into remote woodlands to fight fires. And if you've never had the pleasure of telling your traditionally minded Asian parents that you want to make a living jumping out of airplanes and onto forest fires, let me tell you — it's a magical experience.

It was in this somewhat aimless state that, during my senior year of high school, I joined a rescue squad. Beyond a certain high-mindedness, I didn't really have any specific reasons for doing this. Some of my friends who were already into fire and rescue had some pretty cool stories about their experiences, and ambulance work just seemed somehow more relevant to life than the school athletics I was involved in.

Becoming an emergency medical technician and running ambulance calls, I quickly discovered that I'd gotten myself into a lot more than I'd bargained for. An amazing dichotomy of human existence was becoming evident to me. On the one side, I was seeing the passion, fire, and romance of humanity at heart-rending intensity. (To my gallery of personal heroes, I have added a young man who — with a beautiful wife, a new baby boy, and everything to live for — broke through the barrier of fear built upon the current epidemics of blood-borne diseases to reach out a helping hand, futilely attempting mouth-to-mouth resuscitation on a woman who had gone into cardiac arrest foaming blood at the mouth. At the hospital, samples from the dead woman showed her to have hepatitis. I never learned the fate of that giant among men. I don't even know his name.) On the other hand, I was gaining a gut-level appreciation of the mechanical nature of humans. Spending time in emergency rooms, I've worked with doctors and nurses who could look at a broken person and tell just which parts needed fixing or replacing to make the whole construction get up and walk and talk, once again able to play its part in the human drama.

As I've already mentioned, a lightning strike, eye-opening moment of self-discovery has yet to cement my course in life. I'm finding, however, that the appeal of being a fixer of broken people is growing on me. Pursuing the medical profession isn't a new possibility for me (my parents, for some unfathomable reason, have always wanted me to be a dermatologist), but these days, I'm taking a really careful look at it.

So that's where I am now. My goal in life (or one of my goals, such as they are at the moment) is to be part of the profession that keeps bodies mechanically sound so that their owners can be out doing the dramatic, magical things that make people more than machines. I suppose medical school might figure into this general scheme somewhere — I rather like the idea of becoming a general practitioner and taking care of people's families — but I can also see my life following other avenues in the profession. My goal for the summer, specifically, is to get some experience in the work being done in applied research and to see whether it might be one of these avenues. ■

Clinical Center Proposes Changes in Clinical Protocols Review

Over the past few months, many experts, including the U.S. Inspector General and the External Advisory Committee that reviewed the NIH Intramural Research Program (IRP), have brought to the attention of NIH administrators several deficiencies in the internal quality control and review of clinical protocols at NIH. In May 1994, Michael Gottesman, NIH Acting Deputy Director for Intramural Research (DDIR), assembled a committee of NIH experts to address these concerns. In response to the committee discussions, John Gallin, the newly appointed Director of the Clinical Center and NIH Associate Director for Clinical Research, has recommended changes in the way clinical protocols are reviewed at NIH. The proposed changes are designed to improve the existing review system and at the same time, preserve the independence of ICD research programs.

One fundamental component of the proposed changes is that twice a year, ICDs will receive retrospective and prospective data on protocol performance (for example, patient accrual, including women and minorities, and costs), which they will use to develop processes for retrospective and prospective protocol reviews. The processes for protocol review will be evaluated for review quality by the Clinical Center Director and the NIH DDIR. In addition, the proposed plan stipulates that only patients on active protocols will be admitted to the Clinical Center. This will be ensured by requiring that an active protocol number accompanies all requests for admission and all procedures and tests. These recommendations have been approved in concept by the Institute Directors, the NIH Medical Board, and the Scientific Directors. Each ICD will submit his or her process of protocol review by Sept. 1 to Gallin. The proposed plan is expected to be implemented by the fall of 1994. The measures outlined in the draft proposal are summarized below.

1. All research protocols will be written and be specific for and descriptive of research to be performed. So-called omnibus protocols will be phased out of the NIH Intramural Portfolio of Clinical Protocols at the time of their annual review. Intramural NIH Clinical Portfolios will include three types of protocols: Research, Screening, and Training. Research protocols will include phase I - phase IV clinical trials as well as natural history protocols for studying disease pathogenesis. Research protocols may have multiple components for sceening, drug trials, disease pathogenesis, or long term effects of drugs. Projected and actual patient accrual into these categories must be stated and monitored.

2. All screening and training protocols will be written and will indicate what patients are being screened for. At the time of their annual review, screening protocols will be updated. To identify new syndromes, screening protocols may be written for long-term accrual of cohorts of patients with interesting, unexplained disease presentation. However, the projected number of patients to be accrued must be estimated and then subsequently monitored. The Clinical Center will provide ICD Scientific Directors and Clinical Directors with a prospective estimate of the costs of all screening protocols.

One fundamental change is that twice a year, ICDs will receive retrospective and prospective data on protocol performance which they will use to develop processes for retrospective and prospective protocol reviews.

3. All protocols will be reviewed annually. At the time of the annual review, they will be revised to ensure that any tests being performed are defined.

4. Twice a year, the Clinical Center will meet with the ICD Scientific Directors and the Clinical Director to review all ICD protocols. During these reviews, the Clinical Center will provide each ICD with a list of all active protocols, including the date the protocol was initiated, the projected patient accrual at the time the protocol was initiated and the yearly patient-accrual rate, the percent of women and minorities accrued, the number of NIH employees working on the protocols, and the yearly protocol costs. These details will provide ICDs with the information necessary to conduct retrospective reviews of each protocol. The ICD retrospective review of protocol costs and performance will be reviewed by the Director of the Clinical Center/NIH Associate Director for Clinical Research and the NIH Deputy Director for Intramural Research.

During the biannual reviews, the Clinical Center will also provide a summary table of all new protocols under review or recently initiated. This table will contain projections for patient accrual, a prospective assessment of Clinical Center Departments that will be heavily affected by the protocol, and prospective estimates of the cost of each new protocol. All protocols, including screening protocols, will be updated at the time of the annual review to reflect new areas of research.

Annually, the ICDs will complete a new Intramural Management Controls Evaluation Survey designed to help them comply with all regulations related to clinical research.

5. NIH does not support a rigid quota of patients to be admitted for screening purposes. This may vary widely among ICDs and within an ICD over time. It will be necessary for the use of screening protocols to be justified by the ICDs each year. In this regard, the biannual protocol review will provide ICDs the information needed to ensure that screening protocols are being used appropriately. After these reviews, ICDs will make adjustments in their accrual rates to comply with their goals. The Clinical Center will monitor patient accrual to screening and training protocols to ensure that there is no abuse.

6. The extent of incidental care associated with each protocol will have to be defined. Incidental care will include management of all medical problems that may affect the research protocol or the patient's immediate well-being. At the time of a protocol's annual review, adjustments can be made to accommodate changes in the requirements for incidental care.

7. The NIH Intramural Research Program (IRP) includes Institutional Review Boards (IRBs) for each ICD whose primary mandate is to protect the rights and welfare of human subjects. Protocol review is designed to ensure that risks to subjects are reasonable in relation to anticipated benefits; that selection of subjects is equitable; and that appropriate informed consent is obtained from each prospective subject.

IRBs conduct initial and continuing (at least annual) review and approval of all Clinical Center protocols, including those for screening purposes. Proposed changes in protocol implementation must receive prospective IRB review and approval.

The IRP IRBs function in accordance with the terms and conditions of the NIH Multiple Project Assurance (MPA), the NIH policy document that describes the NIH IRP's compliance with the Department of Health and Human Services' regulations for the protection of human subjects (45 CFR 46). The NIH Office of Human Subjects Research provides oversight of the activities of the IRP IRBs to ensure that they comply with the MPA.

In determining whether an IRB-approved protocol should be implemented, Protocol Implementation Review Committees (PIRCs) are charged with the responsibility for ensuring that

- IRB minutes fully reflect the IRB's deliberations and document review and approval in accordance with 45 CFR 46.
- Where appropriate, additional safeguards have been provided for human subjects, as set forth in 45 CFR 46, subparts A, B, C, and D.
- The protocol is consistent with ICD research objectives and is likely to yield knowledge that will be important to the mission of NIH.
- All collaborative, cooperative, or multisite arrangements, including Cooperative Research and Development Agreements (CRADAs), are fully documented and free of conflict of interest.

Biannual reviews of protocols will be conducted by the Clinical Center and ICD as described below.

8. Two major changes will be made in monitoring protocols to ensure quality control in our policies and procedures.

- As described above, the Clinical Center Director/NIH Associate Director for Clinical Research will monitor patient accrual and resource utilization.
- The Clinical Center Director/NIH Associate Director for Clinical Research and the NIH Deputy Director for Intramural Research will monitor the process by which individual ICDs review the quality of clinical research.
- The Office of the NIH Deputy Director for Intramural Research will implement a new quality-control process, requiring each ICD to complete a new Intramural Management Controls Evaluation Survey to monitor the administration of protocols. This process will help ensure that ICDs are in compliance with all regulations related to clinical research. ■

Cell Cycle Research Interest Group

A Cell Cycle Research Interest Group is being formed to bring together NIH scientists working in this rapidly expanding and exciting area of science. The group plans to invite distinguished speakers from outside NIH, hold seminars on cell cycle research at currently being conducted NIH, and establish biannual poster afternoons to encourage maximum interaction among scientists at NIH and outside. We hope these events will accelerate the dissemination of knowledge, technologies, and cooperation among scientists working in this area. For more information, call Patrick O'Connor, NCI, at 496-3369. ■

NIH Neuroendocrine Immunology Research Interface Study Group Proposed

An NIH-wide neuroendocrine immunology study group is being formed to bring together NIH scientists working on the interdisciplinary areas of psychiatry, neuroendocrinology, endocrinology, rheumatology, and infectious diseases. The goal is to bring about cross-fertilization of ideas, development of collaborations, sharing of information, and joint development of new techniques. The group plans to hold a monthly journal club and research seminar series. If you are interested in joining the club or would like to attend its seminars, fax your request to Esther Sternberg, NIMH, at 402-1561. ■

FIAU Update

Last fall, in the aftermath of the clinical trials of the drug fialuridine (FIAU) to treat hepatitis B patients, NIH and FDA reviewed various aspects of the FIAU studies that resulted in five deaths. In September 1993, FDA held a public hearing, and in November 1993, the agency issued its report on hepatic and pancreatic toxicities associated with FIAU. In May, FDA sent a series of letters criticizing certain aspects of the studies to the principal investigators and drug companies.

However, the NIH Subcommittee to Review FIAU Studies reached different conclusions, as reported on June 2, 1994. After a thorough review of patient records, protocols, and supporting documents, and interviews with principal investigators, staff, and patients, the NIH Subcommittee to Review FIAU Studies, in its report to the Advisory Committee to the Director, NIH, concluded that "there was a justifiable scientific rationale for the NIH FIAU studies" and that "the FIAU studies represent the best of current practice in clinical investigations and exceeded regulatory requirements where such applied." The Subcommittee also concluded that "delayed, fatal human liver toxicity due to FIAU represents a novel type of toxic reaction not previously encountered."

The Subcommittee's report ended with a few specific recommendations on mechanisms of toxicity, preclinical animal tests, patient enrollment, and patient follow-up and its concluding statement recognized the intrinsic risk in conducting clinical trials: "Risk in research cannot be avoided, although we do our best to minimize it. This tragedy will chasten all investigators to remain vigorously alert to the unusual, unexplained or unanticipated in the research process. It also re-emphasizes the importance of understanding risk for their partners in the process — the patients for whom the whole enterprise exists."

In an addendum to the report, the Subcommittee also commented on FDA's letters to the NIH investigators, which indicated a series of failures on the part of the investigators. The Subcommittee recognized that the Institute of Medicine Committee, to be convened in July 1994 by the Assistant Secretary for Health, will need to reconcile the apparent differences in the conclusions of the FDA auditors and the NIH Subcommittee to Review FIAU Studies. Finally, NIH responded to a request from Rep. Edolphus Towns, D-N.Y., Chairman of the Subcommittee on Human Resources of the House Committee on Governmental Operations, by supplying him with copies of documents, patients' records (with personal identifiers masked out), and Subcommittee meeting notes in late June. ■

History of Tyrosine Kinases

by Tony Hunter, Professor of Molecular Biology, The Salk Institute, Recipient of the 1994 Charles S. Mott Prize, General Motors Cancer Research Foundation lectures, June 15, 1994

ABSTRACT:
The phosphorylation of tyrosine residues in proteins was discovered in 1979 during our analysis of the protein kinase activity that phosphorylates the middle T antigen of polyoma virus in vitro. The presence of phosphotyrosine in the middle T antigen was the first indication that tyrosine could be a target for phosphorylation by a protein kinase. Shortly thereafter, we found that v-Src, the Rous sarcoma virus (RSV) transforming protein, and c-Src, its cellular progenitor, also have tyrosine kinase activity. Moreover, we showed that v-Src- transformed cells have elevated levels of phosphotyrosine in protein. Other retroviral transforming proteins were soon found to be tyrosine kinases, and the epidermal growth factor (EGF) receptor was shown to have tyrosine kinase activity that is stimulated by EGF binding.

This evidence immediately implied that tyrosine phosphorylation plays a role in cell growth and transformation. There are now more than 80 known vertebrate tyrosine kinases, and about half of them are receptor tyrosine kinases activated by their ligands. In the past few years significant progress has been made in identifying key substrates for activated receptor tyrosine kinases whose phosphorylation leads to the mitogenic response. These substrates include enzymes, structural proteins, and proteins called adaptors. Most of these substrates have a specialized domain, called Src homology 2 (SH2), that binds to phosphotyrosine in a sequence-specific fashion. Adaptors are SH2 proteins that lack enzymatic activity but that have other protein-binding domains that bind effector proteins. Ligand binding to the extracellular domain of receptor tyrosine kinases induces its dimerization and autophosphorylation, which creates binding sites for cytosolic SH2 adaptor proteins, whose binding and activation triggers signaling pathways leading to the nucleus. One of the best-understood pathways is the Mitogen-Activated Protein (MAP) kinase pathway, in which activated MAP kinase moves into the nucleus, where it phosphorylates and activates transcription factors, leading to gene expression. MAP kinase is activated via a series of protein kinases which is triggered by activated, GTP-bound Ras protein. The loading of GTP onto Ras is stimulated by Sos, a guanine nucleotide exchange factor that is bound to the Grb2 adaptor protein. The binding of the Grb2-Sos complex to a specific phosphotyrosine residue in an activated receptor tyrosine kinase translocates Sos to the membrane, where it can convert Ras-GDP to Ras-GTP. Over the past 15 years, research has revealed that tyrosine phosphorylation, in addition to its role in mitogenic signaling pathways, is involved in many fundamental cellular processes including differentiation, regulation of the cell cycle, and neuronal function.

Splitting hairs: Portrait of Tony Hunter by former NIHer Mark Bitensky suggests the number of known tyrosine kinases may be growing faster than Hunter's beard.

QUESTIONS

Q: *What was your starting point in this research, and how have your questions evolved?*

A: In 1977, we were studying polyoma virus, a small DNA tumor virus that causes tumors in rodents. We were trying to identify the viral gene products responsible for transformation. Interest had centered on a single protein called middle T (or tumor) antigen, which is encoded by one of the three alternatively spliced mRNAs generated from the so-called early region of the polyoma virus genome. As soon as Marc Collett and Ray Erikson, then at University of Colorado in Denver, reported in 1978 that the RSV-transforming protein, v-Src, had protein-kinase activity when assayed in an immunoprecipitate, we began to test whether polyoma virus middle T antigen also had such activity. We were excited to find that middle T antigen became phosphorylated in immunoprecipitates, and by the middle of 1979, we had shown through the use of viral mutants that the presence of this protein kinase activity correlated well with the ability of middle T antigen to transform mammalian cells. In the course of analyzing by acid hydrolysis which amino acid was phosphorylated in middle T antigen, we discovered that the phosphate was not linked to serine or threonine but to another amino acid. We guessed that this might be tyrosine, quickly made some phosphotyrosine, and showed that the product of acid hydrolysis of phosphorylated middle T antigen co-migrated with the synthetic phosphotyrosine. At the outset, we thought this might be a unique property of this viral protein. However, as a control, we were testing v-Src and found that it, too, had tyrosine kinase activity. In this case, we were able to show that c-Src, the cellular progenitor of v-Src, also had tyrosine kinase activity, suggesting that a normal, vertebrate protein could act as a tyrosine kinase. This finding was quickly followed by the demonstration by others that the v-Fps and v-Abl retroviral transforming proteins are tyrosine kinases, and that the EGF receptor also has tyrosine kinase activity that is stimulated by EGF binding. By the end of 1980, we knew of four different cellular tyrosine kinases, and it was apparent that tyrosine phosphorylation played a role in cell growth and malignant transformation. Over the next 15 years, it became apparent that tyrosine phosphorylation functions not only in growth control but also in many other cellular processes, including differentiation, the cell cycle, transcriptional regulation, and synaptic transmission.

Q: *Which findings have been most surprising to you or to other scientists?*

A: Even though phosphorylation of proteins on serine and threonine residues had been known for many years, no one had reported phosphorylation of a protein on tyrosine residues. Thus, it was totally unexpected that the protein kinase activity associated with polyoma virus middle T antigen should be able to phosphorylate tyrosine. The second major surprise was that v-Src and several other retroviral transforming proteins and their cellular counterparts proved to be tyrosine kinases as well. This led to the realization that tyrosine kinases are a major family of cellular enzymes that play important roles in cell growth and many other cellular processes. Indeed, over 80 tyrosine kinase genes are currently known in the human genome, and the true number appears likely to be much larger, once more of the human genome is sequenced.

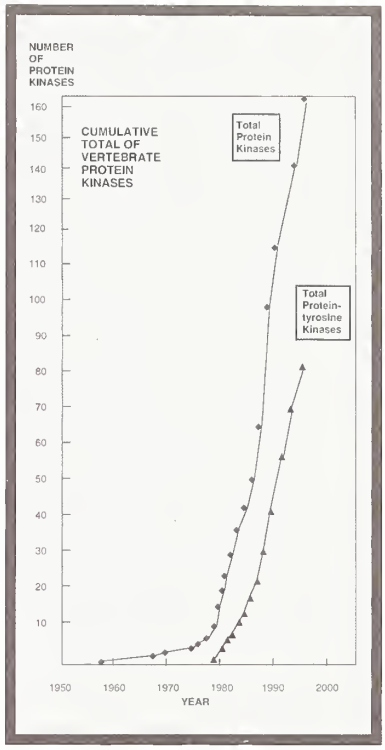
Q: *What were the greatest stumbling blocks, and what new observations, techniques, reagents, or insights helped you get past them?*

A: The initial problem was that there was no source of phosphotyrosine to test the idea that middle T antigen was phosphorylated on tyrosine. At that time, we did not realize that there was a published synthesis for phosphotyrosine, and tried mixing $POCl_3$ with tyrosine. Not surprisingly, we ended up with a black tar, but luckily, we were able to purify a small amount of phosphotyrosine. In fact, we now use an efficient synthesis of phosphotyrosine that had been reported in 1941 by a British biochemist, although why he was making phosphohydroxyamino acids in the depths of World War II is not clear.

The next important technical step was to devise a reliable separation for all three phosphohydroxyamino acids. The separation of phosphotyrosine and phosphothreonine in our original experiments was fortuitous and was due to the use of an old stock of pH 1.9 buffer that had been reused many times for thin-layer electrophoresis. We realized later that upon repeated use, the pH of the buffer drops to 1.7, allowing resolution of phosphotyrosine and phosphothreonine, which comigrate at pH 1.9. Through trial and error, we found a chromatography system that, in combination with electrophoresis at pH 1.7, gave reasonable separation of phosphoserine, phosphothreonine, and phosphotyrosine. This technique, however, was soon superseded by the technique that is still preferred today — a two-dimensional, thin-layer separation using electrophoresis at pH 1.9 followed by electrophoresis at pH 3.5.

The other key problem was to find methods for identifying proteins with phosphorylated tyrosines in the intact cell — the candidate substrates for tyrosine kinases. We developed a method for identifying these proteins based on two-dimensional gel electrophoresis of proteins from ^{32}P-labeled cells. Subsequent treatment of the gel with 1M NaOH hydrolyzes much of the phosphate from phosphoserine but leaves the phosphate on phosphotyrosine, which is rather stable at alkaline pH. As a result, phosphotyrosine-containing proteins are enriched among the remaining ^{32}P-labeled proteins. This technique yielded some of the first substrates for the v-Src tyrosine kinase.

Because the structure of phosphotyrosine resembles that of a dinitrophenyl group — an excellent hapten — an obvious alternative method of finding phosphotyrosine-containing proteins was to try to develop antibodies to phosphotyrosine. We were successful in doing this, but another research group had the same idea and was the first to report the use of anti-phosphotyrosine monoclonal antibodies in detecting phosphotyrosine-containing proteins. Anti-phosphotyrosine antibodies that can be used for immunoprecipitation and immunoblotting have now been developed and are proving invaluable for identifying and analyzing tyrosine kinase substrates.



Q: *Do you see any potential areas where this research might provide insight to clinical scientists?*

A: The clear connection between tyrosine phosphorylation and malignant transformation provided by the RNA and DNA tumor viruses has been extended to human cancer. At least four of the known human oncogenes are mutated forms of tyrosine kinase genes, *Ret*, *Abl*, *Trk*, and the PDGF receptor. The corresponding oncogenes encode tyrosine kinases that are constitutively active. In addition, the EGF and ErbB2 receptor genes, which also encode tyrosine kinases, are commonly amplified and overexpressed in human tumors, and members of the Src protein family often show elevated tyrosine kinase activity in human tumors. For this reason, there has been a strong push to develop specific, low-molecular-weight inhibitors of tyrosine kinases that could be used as cancer therapeutics. A large number of inhibitors have been reported in the past 15 years, and some of these appear to be reasonably specific. One inhibitor that is specific for the EGF receptor has just been described and appears to have some inhibitory effect on the growth of human tumor cells that express high levels of the EGF receptor in mice. Several companies have active programs aimed at developing drugs targeted at specific tyrosine kinases involved in cancer and other human diseases.

Q: *What does your work focus on now, and what questions would you ultimately like to answer?*

A: My lab's work is now focused largely on the signal pathways activated by receptor and nonreceptor tyrosine kinases. Ligand-induced receptor auto- phosphorylation creates binding sites for substrates and target proteins that have SH2 domains. SH2 domains bind to phosphotyrosine in a sequence-specific manner. Thus, following ligand binding, the activated receptor tyrosine kinase binds a set of SH2-containing proteins that recognize the individual phosphorylation sites displayed by the receptor in question. These SH2 proteins are activated by phosphorylation, by binding to the receptor, or by translocation to the membrane. Some substrates are enzymes, such as phospholipase Cg, and others are structural proteins. We are concentrating on a group of targets known as SH2 adaptor proteins which lack enzymatic activity but contain additional protein-interaction domains, such as SH3, that bind effector proteins that propagate the signal. Specifically, we are trying to elucidate the roles that Grb2 and Nck play in mitogenic signaling. We are also studying protein-tyrosine phosphatases — enzymes that reverse tyrosine phosphorylation — because it is clear that these enzymes must also be essential for any process regulated by tyrosine phosphorylation. ■

Attention Postdocs/Clinical Fellows: There are fellows working to improve your experience at NIH. For more information on the NIH postdoctoral Fellows Committee you can visit the Office of Education's booth at the NIH Research Festival. ■

HOT METHODS CLINIC:
THE FAST TRACK TO COMPLETE cDNAs: 5' RACE

by Richard E. Manrow, Ph.D., NCI, and Lance Liotta, M.D., Ph.D., NCI

The ability to rapidly amplify cDNA ends (RACE) has accelerated the pace at which complete cDNA sequences can be obtained. This method has greatly facilitated protein sequence determination and mRNA structure, sequence, and expression studies. Useful information can now be obtained quickly, even from low-abundance mRNAs. Currently, investigators can analyze messages in samples of fewer than 100 cells; in the future, detailed analyses may be possible for the study of individual cells.

The Method and How it Works

Complete cDNA and protein sequence information is essential for structural and expression studies and, ultimately, for the isolation and characterization of genomic clones. Unfortunately, the cDNAs obtained from most libraries are incomplete. Most frequently, they lack sequences found at the extreme 5' ends of their mRNA templates. The principal reason for this is that reverse transcriptases (RTs), used to create cDNAs from mRNA templates, often fail to traverse entire mRNA molecules during cDNA synthesis. Until the development of 5' RACE technology, investigators had no choice but to re-screen libraries to obtain other cDNA clones that contained the missing information. These re-screening efforts were tedious and not always successful.

The solution to this problem was simple and elegant: existing methods for adding known sequences to the 3' ends of single-stranded DNAs were coupled with the polymerase chain reaction (PCR) to selectively amplify specific cDNA targets. As is often the case when scientists are trying to solve a critical problem, more than one approach was explored. The two 5' RACE strategies that emerged (1-3) are outlined in Figure 1. In both cases, the first step involves cDNA synthesis using an oligonucleotide primer (G1) that anneals to the known mRNA template for the sequences to be extended. Most investigators employ either avian myeloblastosis virus (AMV) RT or Moloney murine leukemia virus (MMLV) RT to accomplish this. However, a DNA polymerase from the thermophilic bacterium *Thermus thermophilus* has recently been shown to exhibit a strong RT activity in the presence of Mn^{+2} (4); use of this enzyme (Tth DNA polymerase) at elevated temperatures (i.e., 70 °C) may enhance the likelihood that cDNA synthesis will not terminate prematurely on GC-rich templates or at sites possessing secondary structure (5).

The two 5' RACE strategies diverge at the next step, in which specific DNA sequences are linked to the 3' ends of the newly synthesized cDNAs in order to mark them for amplification. In one approach, homopolymeric tails are added using the enzyme terminal deoxynucleotidyl transferase (terminal transferase, or TdT). Subsequently, PCR amplification of the tailed cDNA is performed using a second gene-specific primer (G2; nested with respect to the cDNA primer) and a mixture of two other primers, each bearing the same unique sequence (an arbitrary "anchor" sequence determined by the investigator) and one of them bearing, in addition, a homopolymeric tail complementary to the tails added to the cDNA. In this way, the sequence of interest becomes flanked by unique sequences (i.e., a known cDNA sequence and the anchor sequence), thereby marking it as a target for selective amplification. Use of anchor sequences in the upstream primers is important because homopolymeric primers may anneal non-specifically to sequences other than the desired ones during PCR. Use of nested, gene-specific primers in the amplification phase also serves to increase specificity.

In the second approach, an anchor oligomer is ligated directly onto the 3' ends of the newly synthesized cDNAs using T4 RNA ligase. This approach takes advantage of observations made by Tessier et al. (6) that T4 RNA ligase will ligate two single-stranded DNA segments in the presence of hexamine cobalt chloride. Even under optimum conditions, this ligation reaction does not go to completion, but efficiencies of 40 - 60% have been reported. Given the amplifying power of PCR, even limited anchor oligomer ligation should be sufficient; however, the following precautions must be taken to ensure that the desired ligation reaction is favored and that self-ligation of the anchor oligomer or the cDNA is minimized: 1) the cDNA primer cannot have a phosphate group at its 5' end; 2) the anchor oligomer must be phosphorylated at its 5' end by using T4 polynucleotide kinase; and 3) the 3' end of the anchor oligomer must be blocked by the addition of a ddAMP moiety (or a methyl group).

The reactions unique to each of these two 5' RACE strategies work sufficiently well that commercial kits have been built around them. The 5' RACE System Kit sold by Life Technologies, Inc. (GIBCO-BRL), uses a variation of the homo-polymeric tailing method; the 5'-ampliFINDER RACE Kit from Clontech Laboratories, Inc., employs the anchor oligomer ligation method. Because some scientists dislike using kits, detailed protocols for the described reactions are provided below. Please note that these protocols and those found in the kits may differ in some aspects. Mention of specific products does not constitute an endorsement.

Protocols

Single-Stranded cDNA Synthesis and Purification.

cDNA synthesis is usually performed using 1 - 2 µg poly(A)$^+$ RNA as the template; however, successful 5' RACE has been achieved with less than 1 ng total cellular RNA (3). The G1 oligonucleotide used to prime cDNA synthesis should be approximately 20 residues long and have a GC content of 45 - 65%. Use sterile, RNAse-free water to resuspend and dilute the G1 oligomer; the other reaction components should also be RNAse-free. Typically, reaction mixtures range in volume from 20 to 40 µL. Prior to setting up the final mixture, the RNA template, the G1 primer, and the aqueous component of the mixture should be combined in a sterile tube, heated at 65 °C for 5 min., and then chilled on ice.

The reaction conditions using AMV RT are

- ≤1 - 2 µg RNA template
- 10 pmol G1 primer
- 50 mM Tris-HCl, pH 8.3
- 50 mM KCl
- 10 mM $MgCl_2$
- 1 mM dNTPs (Na^+)
- 1 mM DTT
- 1 mM EDTA
- 4 mM sodium pyrophosphate
- 10 µg/mL bovine serum albu min (BSA)
- 40 units placental RNAse inhibitor (RNAsin; Promega Corporation)
- 10 units AMV RT
- Incubate at 42 °C for 1 hour, followed by an optional 30 min. incubation at 52 °C.

The reaction conditions using MMLV RT are

- ≤1-2 µg RNA template
- 10 pmol G1 primer
- 50 mM Tris-HCl, pH 8.3
- 75 mM KCl
- 10 mM DTT
- 3 mM $MgCl_2$
- 0.5 mM dNTPs (Na^+)
- 100 µg/mL BSA
- 40 units RNAsin
- 200 units MMLV RT
- Incubate at 42 °C for 30 min..

The reaction conditions using Tth DNA polymerase RT are

≤1-2 µg RNA template
10 pmol G1 primer
10 mM Tris-HCl, pH 8.3
90 mM KCl
1 mM $MnCl_2$ (optimization may be necessary)
0.2 mM dNTPs
5 Units Tth polymerase (available from Perkin Elmer or Epicentre Technologies)
Overlay the reaction mix with mineral oil to prevent evaporation and incubate at 70 °C for 15 min..

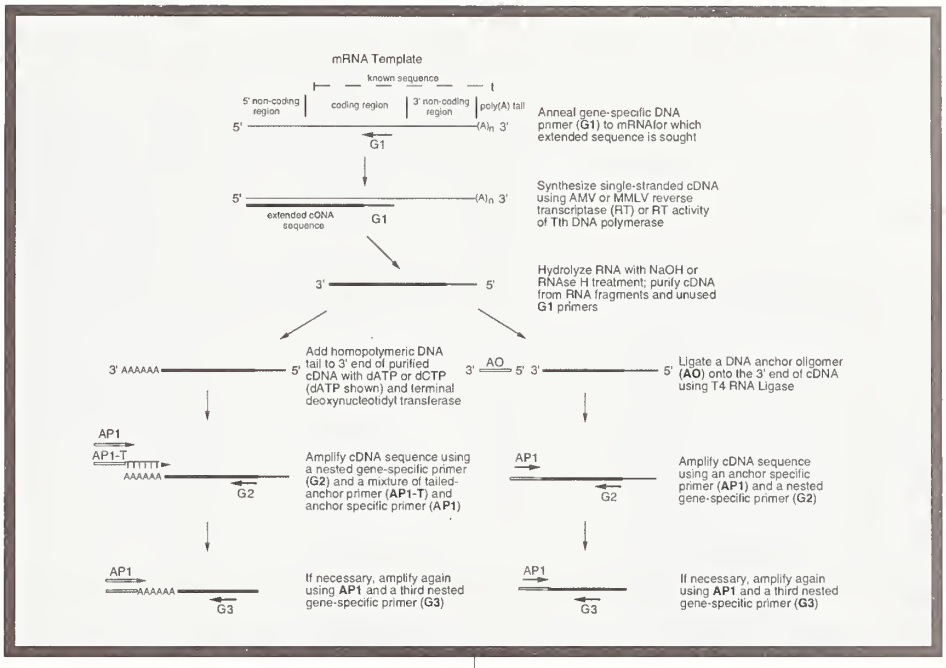
The cDNA synthesis reactions are terminated by adding EDTA to a final concentration of 15 mM (for AMV RT and MMLV RT) or by adding EGTA to a concentration of 0.75 mM (for Tth DNA polymerase). The RNA template is hydrolyzed (and the enzymes denatured) by adding NaOH to a concentration of 400 mM and incubating the mixture at 65 °C for 30 min. Acetic acid is then added to a concentration of 400 mM to neutralize the solution. Some investigators terminate MMLV RT reactions and simultaneously inactivate the enzyme by heating the reaction mix at 65 - 70 °C; they then hydrolyze the RNA template by treatment with *Escherichia coli* RNAse H (2 units per reaction tube). The cDNA is seperated from unused G1 oligomers and residual RNA by differential binding to a silica matrix (GENECLEAN, Bio101 Inc., or similar matrices included in the 5' RACE kits described above) in the presence of 4.0 - 4.5M NaI. The bound cDNA is washed as recommended by the vendors and eluted with sterile, distilled water. Extreme care must be taken to avoid silica contamination of the eluted material. The volume of the cDNA sample should be adjusted to approximately 10 µl. Other approaches have been used to deal with residual G1 oligomers, ranging from doing nothing at all to removing them either by size exclusion chromatography or gel electrophoresis. The presence of unused G1 oligomers may interfere with subsequent amplification reactions.

Homopolymeric cDNA Tailing.

The sample of cDNA should be heated at 90 °C for 2 min. to remove secondary structure and then chilled on ice. All or part of the cDNA may be tailed. Tailing reactions are usually performed at 37 °C for 5 min. in 20 - 25 µL reaction mixtures containing

cDNA
100 mM potassium cacodylate, pH 7.2
2 mM $CoCl_2$
0.2 mM DTT
0.2 mM dATP (or dCTP)
10 units TdT.

The tailing reaction is terminated by heating the mixture at 70 °C for 5 - 10 min. At this point, the tailed cDNA may be diluted ~25-fold with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), or it may be precipitated with ethanol, using 20 µg of glycogen as the carrier. If the cDNA is precipitated, resuspend it in approximately 500 µL sterile, distilled water. Typically, 1 - 2% of the tailed cDNA is used in amplification reactions.



5' End Phosphorylation of an Anchor Oligomer.

Prepare a 40-µL reaction mixture containing

2 nmol anchor oligomer (usually 35 - 45 nucleotides long; sequence determined by the investigator or kit manufacturer)
70 mM Tris-HCl, pH 7.5
10 mM $MgCl_2$
1 mM DTT
0.5 mM ATP
20 Units T4 polynucleotide kinase.

Allow the phosphorylation reaction to proceed at 37 °C for 60 min., and then heat-inactivate the kinase by incubating the reaction tube in a 65 - 70 °C water bath for 20 min. Precipitate the phosphorylated oligomer with ethanol, resuspend it in sterile, distilled water (up to 50 µL), and block the 3' end of some or all of it with ddATP and TdT as described below.

3' End Blocking of an Anchor Oligomer with ddATP.

A 70 µl reaction mixture is prepared containing

1 nmol anchor oligomer
0.2 mM ddATP
100 mM potassium cacodylate, pH 7.2
2 mM $CoCl_2$
0.2 mM DTT
10 Units TdT.

The mixture is incubated at 37 °C for 60 min. The reaction is terminated by adding 210 µL of ice-cold 20 mM EDTA, and the blocked oligomer is precipitated with ethanol and resuspended in sterile, distilled water.

Anchor Oligomer Ligation to cDNA.

All or part of the cDNA prepared above may be ligated to the anchor oligomer. Since the final volume of the ligation mixture is 10 µL, the cDNA sample may have to be concentrated by vacuum drying. The ligation reaction is performed at room temperature for 12 - 24 h. with the following components:

cDNA sample prepared above
1-10 pmol 5' phosphorylated, 3' blocked anchor oligomer
50 mM Tris-HCl, pH 8.0
10 mM $MgCl_2$
1 mM hexamine cobalt chloride
20 µmol ATP
10 µg/mL BSA
25% (w/v) PEG 8000
10 units T4 RNA ligase.

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Recently Tenured

Juan S. Bonifacino *received his Ph.D. from the University of Buenos Aires, Argentina, in 1981. He joined the Cell Biology and Metabolism Branch (CBMB), NICHD, in 1984 and currently heads its Unit on Intracellular Protein Trafficking.*

Over the past few years, I have been interested in various aspects of the biogenesis and transport of integral membrane proteins within the secretory pathway. Many of my studies have examined the relationship between protein structure and intracellular trafficking. Research in my group focuses on two main topics that exemplify different mechanisms of protein localization within cells: 1) the assembly of multisubunit complexes in the endoplasmic reticulum (ER) and the retention of incompletely assembled complexes within the ER, and 2) the mechanisms of protein localization to a specific compartment of the secretory pathway, the trans-Golgi network (TGN).

My interest in the assembly of multisubunit complexes stems from work I did as a postdoctoral fellow in Richard Klausner's laboratory at NICHD. Our early studies of the assembly of the T-cell antigen receptor showed that only completely assembled complexes were efficiently transported to the cell surface, whereas free chains and incomplete complexes were largely retained intracellularly and in some cases, degraded. These phenomena have now been observed for many other protein complexes, but the molecular mechanisms involved are not well understood. Recent work from my laboratory on another family of hetero-oligomeric complexes — class II antigens of the major histocompatibility complex (MHC) — has provided insights into the early events in protein assembly in the ER and the mechanisms that control transport of newly made complexes from the ER. We found that incompletely assembled class II MHC molecules form large, heterogeneous aggregates in association with the ER chaperone, immunoglobulin-heavy-chain binding protein (BiP). Formation of such aggregates may be a determining factor in the process by which unassembled subunits are retained in the ER. Strikingly, we also found that BiP-associated aggregates exist transiently during assembly of normal class II MHC molecules in spleen cells. This suggests that aggregates are not necessarily aberrant products but are most likely true intermediates in the normal assembly process. These and other observations have allowed us to establish a sequence of events in the assembly of class II MHC molecules.

Whereas retention of unassembled subunits in the ER may depend on the general physicochemical properties of the proteins (i.e., aggregation and association with ER chaperones), localization to other compartments of the secretory pathway appears to be mediated by more specific signals. Work in my laboratory has led to the identification of a signal that mediates protein localization to the trans-Golgi network. The signal is borne within the cytoplasmic domain of two TGN-specific proteins, TGN38 and furin, and consists of a tyrosine-based motif related (but not identical) to internalization signal sequences. These observations, together with similar findings in yeast proteins, suggest the existence of a general mechanism for protein localization to the TGN that relies on specific recognition of cytoplasmic signals. We are now conducting studies to identify molecules that interact with such signal sequences and that control protein localization to the TGN.

Pim Brouwers *received his Ph.D. from McGill University, Montreal, in 1979. He came to NIH from Georgetown University in 1988, joining the Pediatric Branch, NCI, where he currently heads the Neuropsychology Group.*

Our laboratory studies the neurobehavioral consequences of chronic illness and its treatment in children and adults, particularly in patients with cancer and HIV infection. Our main efforts focus on characterizing disease-specific abnormalities in intellectual, mnemonic, attentional, socioemotional, and judgmental abilities and on documentating treatment-related changes in these functions.

Using existing test instruments and newly developed approaches, we can now comprehensively characterize central nervous system (CNS) manifestations and sensitively document treatment-related changes in neurobehavioral functioning. We then try to establish that these neurocognitive scores are associated with physiologic changes in brain images, cerebrospinal fluid (CSF), or laboratory markers that indicate abnormalities in the CNS resulting from disease or treatment. This is important in validating neurocognitive measures as markers of the effects of the disease on the CNS and ruling out other confounding factors (e.g., emotional and socioeconomic factors).

In this way, we have established that long-term survivors of childhood acute lymphoblastic leukemia (ALL) who received cranial irradiation as preventive therapy experienced adverse late sequelae both on intellectual and computed tomography (CT) brain-scan tests. We also showed that the effects on the intellect were associated with abnormalities in the CT scan, indicating that the neurobehavioral sequelae in ALL have an organic basis. We are currently evaluating long-term survivors who received less neurotoxic but equally protective CNS therapies.

We have also developed a technique for measuring the incidence and severity of CT brain-scan abnormalities in HIV infected children. We found that neurocognitive deficits and aberrant behavior are related to the degree of CNS abnormality, establishing the clinical significance of these lesions. Additional quantitative and longitudinal studies using magnetic resonance imaging (MRI) technology are under way. We also analyzed the CSF of children with symptomatic HIV infection for the possible presence of neurotoxins and found elevated concentrations of quinolinic acid (QUIN) that inversely correlate with the level of neu-

ropsychological functioning. In ongoing studies, we are exploring CSF-to-serum ratios of QUIN and changes with therapy. A relation between disease stage, defined by CD4 measures and P24 levels, and CNS structure and function, indicated that advanced disease puts children at higher risk for significant HIV-associated CNS manifestations. Longitudinal studies that follow individuals over time and that use multiple regression models are in progress.

We further developed a methodology for evaluating neurobehavioral changes in chronically ill patients who are undergoing treatment in clinical trials. We were the first to show significant improvements in neurocognitive function with 3'-azida-3'-deoxythymidine (AZT) therapy in adult patients with AIDS dementia complex. We later extended these findings to children with HIV infection where significant improvements were observed in both encephalopathic and nonencephalopathic patients. Concurrent decreases in the size of enlarged ventricles and subarachnoid spaces on CT scans validated these findings. In addition, we observed a decrease in CSF QUIN with a concurrent increase in general cognitive function. In ongoing clinical trials, we have demonstrated and are further exploring pharmacokinetic correlations, relating changes in neurobehavioral function to dose effects, absorption area under the curve of the antiretroviral agent, and the agent's penetration into the CNS.

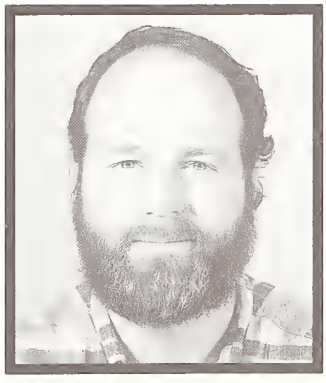
Jean Lud Cadet *received his M.D. from Columbia University in New York in 1979. He joined the Clinical Pharmacology Branch, NIDA, in 1992 as the Chief of the Unit on Neuropsychiatry and Neurotoxicology. He currently heads the Molecular Neuropsychiatry Section in the Neuroscience Branch at NIDA.*

Researchers in my section are interested in the cellular and molecular mechanisms of development, neurotoxicity, and neurodegeneration. Our basic hypothesis is that oxygen radicals and other free radicals play an important role in these processes.

During cellular metabolism, aerobic organisms generate oxygen-derived free radicals, including superoxide and hydroxyl radicals. These substances can cause lipid peroxidation and oxidation of biomolecules, and are thought to be involved in myocardial infarction, strokes, and the neurodegenerative aging process. But aerobic organisms have evolved mechanisms that enable them to survive in the face of the ubiquitous presence of these free radicals. These mechanisms involve enzymes such as superoxide disumutase (SOD) and glutathione peroxidase. During the past few years, we have been studying the role of superoxide radicals in drug-induced neurotoxicity. Using transgenic mice that express elevated levels of human CuZnSOD, we have shown that these SOD Tg mice are protected against the neurotoxicity of N-methyl-4-phenyl-1,2,3,6-tetra-hydropyridine and methamphetamine — compounds that are dopamine neurotoxins in mice. These results suggest that the neurotoxicity of these drugs is associated with superoxide radical formation.

We are also interested in the role of nitric oxide in the neurotoxicity of drugs of abuse. Using primary cultures from rat fetal mesencephalon, our laboratory has recently demonstrated for the first time that inhibition of nitric oxide synthase can attenuate the neurotoxicity of methamphetamine. We have also demonstrated for the first time that inhibitors of ADP-ribosylation can also protect against the toxicity of this drug in vitro. In addition, we have been able to establish a model for drug-induced gliosis in vitro. Using that model, we showed that inhibitors of ADP-ribosylation can prevent reactive gliosis. Using both in vitro and in vivo model, and several probes, we are continuing to dissect the specific pathways involved in neuronal cell death. We are also interested in finding out whether these same pathways are involved in apoptosis, or programed cell death.

Byron Caughey *received his Ph.D. from the University of Wisconsin-Madison in 1985. He came to the Laboratory of Persistent Viral Diseases (LPVD), Rocky Mountain Laboratories, NIAID, in 1986 from Duke University in Durham N.C., and will be a research chemist in LPVD.*



My lab focuses on transmissible spongiform encephalopathies (TSEs), which are infectious and fatal neurodegenerative diseases occurring most prominently in sheep (scrapie), cattle (mad cow disease), and humans (kuru and Creutzfeldt-Jakob disease). The obscure infectious agent of these diseases resembles a virus in that it replicates in the host and has distinct strains, but so far, no agent-specific nucleic acid has been identified. The unusual biochemical properties of the agent led almost three decades ago to still-unproven hypotheses that it contains only protein. More recently, this putative "infectious protein," or "prion," was proposed to be PrP-res (or PrPSC), a neuropathogenic, abnormally protease-resistant and amyloidogenic form of a host-encoded protein, PrP. We are interested primarily in how PrP-res is made, how its formation might be blocked, and what relationship its formation has to TSE-agent replication and pathogenesis.

Using scrapie-infected tissue culture cells developed by my LPVD colleagues Rick Race and Bruce Chesebro, my lab established that PrP-res is derived posttranslationally from normal, protease-sensitive PrP (PrP-sen) and that the subcellular site of conversion is the plasma membrane and/or along an endocytic pathway leading to the lysosomes. We also used these cells to identify some potent and selective inhibitors of PrP-res accumulation and scrapie-agent replication. These inhibitors appear to act by competitively inhibiting an interaction between PrP-res and an endogenous sulfated glycosaminoglycan (GAG). GAGs are components of pathogenic amyloids associated with many diseases, and our studies suggest that GAG-amyloid interactions may be attractive targets in designing drugs for these dis-

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Commentary

Potential of Pharmacologic Therapy for Enhancing Collateral Growth in the Ischemic Heart

by Ellis Unger and Stephen E. Epstein (Senior Research Investigator and Chief, respectively, Cardiology Branch, NHLBI)

Richard Lower provided the first description of coronary collaterals in humans in 1669, when he noted that fluid injected into one coronary artery emerged from another. We now know that coronary collaterals exist at only a rudimentary stage of development in the normal heart; however, with the hemodynamic alterations and/or metabolic derangements that accompany occlusive coronary artery disease, their development is stimulated, and they provide an alternative source of myocardial perfusion to the territory of the impeded artery.

The presence of functional collaterals enables the heart to maintain essentially normal perfusion under resting conditions, despite near-total occlusion of a major coronary artery. During the stress of exercise or the infusion of a vasodilator, however, the limitations of these vessels become apparent: vasodilator reserve is attenuated, maximal perfusion is curtailed, and myocardial ischemia results if the metabolic demands of the heart are unmet. The importance of coronary collaterals is underscored by the demonstration that the degree of myocardial dysfunction that develops in patients following acute occlusion of a coronary artery (during balloon angioplasty, for example) is inversely related to the extent of collateral vessels present (1). Moreover, myocardial viability in patients with recent myocardial infarction (MI) is correlated with the extent of collateral blood flow within the territory of the infarct-related artery (2).

Given the clinical importance of collaterals, two important questions emerge: what factors lead to their development, and what is the possibility that their growth can be enhanced pharmacologically? These questions have been a focus of the Physiology and Pharmacology Section of the Cardiology Branch, and based on our investigations in an animal model, we have now concluded that coronary collateral growth can be stimulated by using angiogenic peptides, at least in experimental animals (3,4).

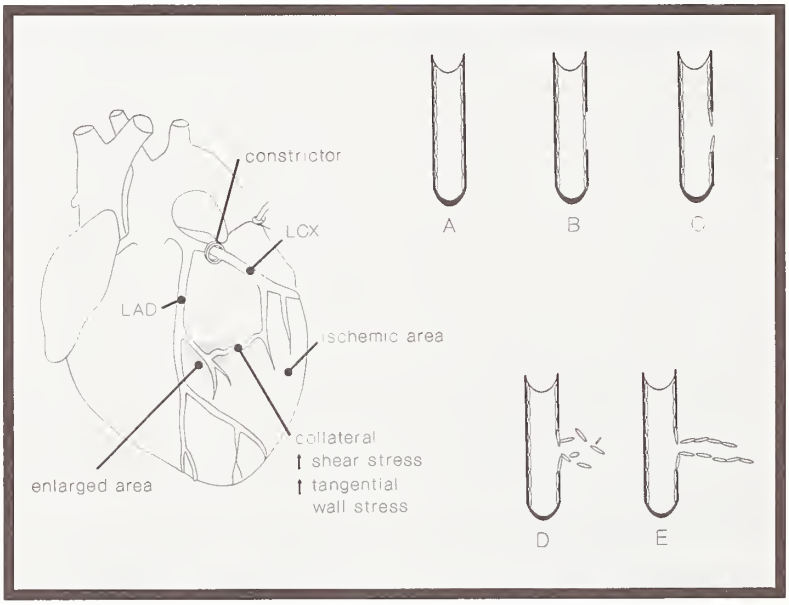

Figure 1. Left: Schema of experimental model used to engender coronary collateral formation. A constrictor placed on the left circumflex coronary artery (LCX) causes progressive obstruction of the proximal vessel. With the decline in LCX flow, there is the potential development of ischemia in the LCX territory, as well as a pressure gradient between the LCX territory and other normally perfused regions of the heart. In the shaded area, a collateral vessel is depicted, having developed between the branches of the LCX and the left anterior descending coronary artery, the other principal coronary artery of the dog. Stimuli for angiogenesis may originate from within the vascular wall (i.e., shear stress and tangential wall stress), the ischemic myocardium, or both. Right: Stages of angiogenesis are represented from the shaded area (enlarged) on the left: A) quiescent vessel, B) degradation of basement membrane, C) endothelial cell migration, D) further endothelial cell migration and proliferation, and E) tube formation. Later, the collateral will become invested with layers of vascular smooth muscle cells.

"Angiogenesis" refers to the growth and/or development of blood vessels. In 1971, in the course of exploring the mechanisms responsible for vascular growth in malignant tumors, Judah Folkman and his colleagues discovered a diffusible substance that stimulated the growth of blood vessels (5). Subsequently, Schaper and co-workers provided support for the concept of a diffusible mediator of collateral growth in the heart (6). In 1985, Vallee et al. were the first to purify a human angiogenesis factor (angiogenin), and shortly thereafter, several other angiogenic peptides were isolated (see below).

The stimuli leading to the synthesis, release, and activation of angiogenic factors are probably multiple. Evidence suggests that ischemia itself can provide adequate stimulus for vascular growth; however, a competing (but not mutually exclusive) theory is that mechanical or hemodynamic factors initiate collateral development. In the normal heart, there may be many immature, undeveloped anastomoses between coronary arteries that carry little, if any, perfusion in the absence of a pressure gradient driving flow across them. With the development of proximal obstruction in one of the coronary arteries, there is the simultaneous development of a pressure gradient between the nonobstructed and obstructed arteries, inducing flow across the collateral vessel (Fig. 1). With the increase in flow, augmented collateral shear stress and tangential wall stress (stretch) may be important triggers in the initiation of angiogenesis (7). We think it is likely that both metabolic and hemodynamic factors are responsible for initiating and maintaining collateral development, and the cellular, biochemical, and molecular events that transduce these influences into vascular growth are currently under intense investigation.

In early 1985, we embarked on a series of experiments in which we hoped to facilitate the development of coronary collaterals by using angiogenic substances. We thought it likely that growth factors could serve as endogenous biological "distress signals," initiating the angiogenic response. Such mediators might be synthesized or released by the vascular wall and/or the myocardium in response to the development of significant coronary artery occlusions. Given the imperfect nature of collaterals — that is, their inability to provide adequate perfusion under conditions of stress — we then hypothesized that exogenous administration of such mediators, adding to endogenous stores, might enhance angiogenesis and improve myocardial perfusion. Initially, these hypotheses were difficult to test because 1) the angiogenic polypeptide growth factors had not been well characterized, 2) there was no practical method for administering growth factors to the heart, and 3) the assessment of biological endpoints relevant to angiogenesis was difficult.

Several polypeptide growth factors were eventually characterized and purified, including acidic fibroblast growth factor (FGF), basic FGF, vascular endothelial growth factor (VEGF), insulin-like growth factor I, scatter factor (hepatocyte growth factor, a glycoprotein), and others. Acidic FGF, basic FGF, and VEGF became available in large quantities through recombinant-DNA technology, making possible

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COMMENTARY

SILICONE GELS INDUCE PLASMACYTOMAS IN BALB/C MICE

by Michael Potter, Chief of the Laboratory of Genetics, NCI

In January 1992, David A. Kessler, Commissioner of the Food and Drug Administration (FDA) requested a voluntary moratorium on the clinical use of silicone-gel-filled breast implants until more was known about the biological activity of the components (1). Our laboratory had been studying materials such as paraffin oils and solid plastics that induce the formation of plasmacytomas (PCTs) in genetically susceptible strains of mice, and the FDA moratorium rekindled our interest in exploring silicone as another possible plasmacytomagenic material. Our studies now show that injection of silicone gel into the peritonium of Balb/c mice induces plasmacytoma formation.

As a poorly digestible chemical, the physically stable silicone gel represented a new type of material that would not produce the mechanical effects of the solid plastics or have the liquidity of the paraffin oils, such as pristane, which we had studied extensively. The chemistry of silicones and, particularly, the complex silicone gels, could open new possibilities for studying the tumorigenic properties of these agents, which have similarities to solid-state carcinogens (2). In general, such agents evoke chronic inflammatory reactions.

Plasmacytoma Induction

Silicone gels were obtained from mammary implants purchased commercially. Manipulation of these gels was difficult because they are highly sticky, very elastic, and could not be cut into fragments and inserted into the peritoneum. Each time a small amount was pushed into the space and the instrument withdrawn, the gel came out with it. Susan Morrison in our lab, however, developed a simple method for injecting this gel into mice (3). BALB/c mice began developing PCTs around 5 months after the first of either one 0.4-mL or three 0.1-mL injections of the gel; in these experiments, the yield of PCTs in groups of mice was comparable to that obtained with single 1.0 -mL or three 0.5-mL injections of pristane (3). Further work is in progress to compare these two agents. The silicone gels have not yet produced PCTs in other strains of mice, nor have they induced PCTs when injected subcutaneously in BALB/c mice. We have carried out some very preliminary work with linear dimethylpolysiloxane (DMPS) polymer, but this liquid form of silicone has not yet produced PCTs, either. Thus far, we have results with gels from two implants, and one is only half as effective as the other in generating PCTs. We are now testing more gels, including preparations that can be made in the laboratory.

The injected silicone gel congeals into a single mass in the abdominal cavity. As long as 5 to 10 months after injection, the blob of gel remains discrete and can be lifted out of the peritoneum intact, but after 13 months, the gel is broken down, and all that remains is stringy, sticky material. Injected mice do not develop ascites, and the vast majority of the peritoneal surfaces appear normal. The gel blobs appear to be well tolerated and relatively noninflammatory, but later, cells infiltrate some of them, changing them from clear to cloudy. In contrast to the response to pristane, injection with silicone does not affect the diaphragm and upper abdominal connective tissues; however, the gel is a source of liquid material that seeps out from the blob into the peritoneal space. This liquid material provokes the formation and deposition of a granulomatous tissue that accumulates with time on the intestinal mesenteric surfaces and in the omentum. Small spheres of oily material become surrounded by inflammatory cells in the peritoneal space, and these aggregates adhere to peritoneal surfaces and then become organized into a silicone granulomatous tissue. Much of the process of plasmacytomagenesis takes place in this tissue.

The silicone-granulomatous tissue consists of highly refractive spaces or vacuoles where liquid silicone material has been deposited. During the first 6 to 8 months after injection,the silicone granulomatous tissue has a high content of inflammatory cells that accumulate between the vacuoles. These cells include macrophages, multinucleated giant cells, fibroblasts, neutrophils, lymphocytes, plasma cells, and probably other cell types. Later, the granuloma changes. In some, but not all mice, there is a dense deposition of collagenous material around the vacuoles. Quite late, after a year, the granulomatous tissue is dramatically different: the large vacuoles break down, giving way to many smaller ones, creating a foamy appearance. The inflammatory cells largely disappear, and at this stage, the silicone granuloma seems to be "burned out".

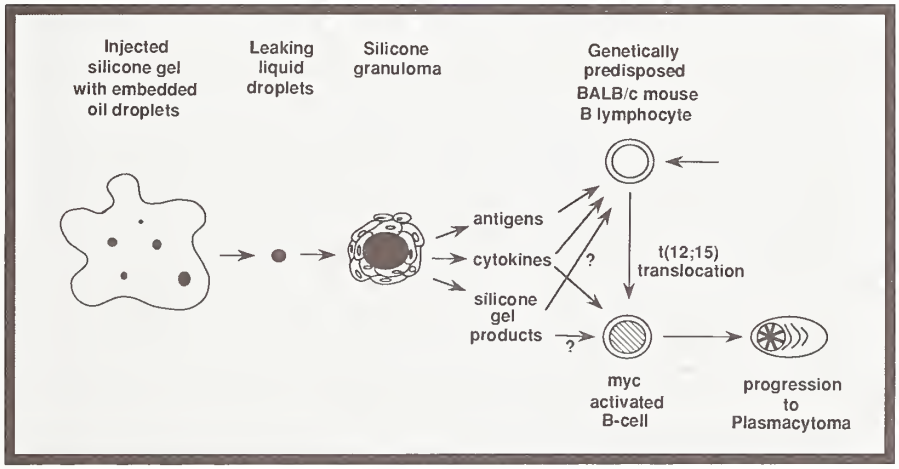

Figure 1. Hypothetical scheme of how silicone gels may interact with B-cells. Oily liquid materials trapped in the gel or derived from it leak out and interact with cells such as macrophages. This interaction yields antigens, cytokines, and possibly chemical products from the silicone that interact with B-lymphocytes and/or B-cells carrying t(12; 15) translocations. These stimuli may provide essential factors that permit B-cells to progress to plasmacytomas.

The Importance of the Peritoneal Site

The peritoneal connective tissues appear to be a required site for pristane- and silicone-induced PCT development in mice. There are several possible reasons for this. First, the peritoneal space has a cadre of resident macrophages, and when stimulated by materials such as pristane, large numbers of new cells of the monocyte and neutrophil series migrate into the space to engage and remove the oil droplets. Second, the intestinal mesenteric vessels appear to undergo angiogenesis and supply the newly developing tissue. Third, the vascular supply also serves as a route for circulating B lymphocytes to enter the granulomatous tissue. Histological sections taken at various times after the injection of silicone or pristane show various kinds of plasma cell proliferation that progressively becomes larger and contains more atypical cells that resemble those seen in fully developed PCTs.

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POTENTIAL OF PHARMACOLOGIC THERAPY
continued from page 16.

studies of their effects on coronary angiogenesis.

Our recent studies have focused on two peptides: basic FGF and VEGF. Basic FGF is the most extensively characterized member of the FGF family, a group of angiogenic heparin-binding polypeptides. This growth factor is produced by diverse cell types, including endothelial cells and cardiac myocytes. Basic FGF stimulates the proliferation of cells of mesodermal and neuroectodermal origin, targeting vascular endothelial cells, fibroblasts, smooth muscle cells, neuroblasts, osteoblasts, and melanocytes (8,9). VEGF is an angiogenic dimeric peptide with sequence homology to the A and B chains of platelet-derived growth factor (10). It was independently isolated as (and is identical to) vascular permeability factor (VPF). Its potency as an inducer of permeability exceeds that of histamine by orders of magnitude in some systems. It is functionally similar to basic FGF in its ability to stimulate the proliferation of vascular endothelial cells and induce angiogenesis; however, it differs from basic FGF in that its trophic effects are specific for endothelial cells.

Initial published reports suggested that the half-life of basic FGF was approximately 2 min. after intravascular administration. On the basis of this observation, we believed it necessary to administer basic FGF and other growth factors on a continuous basis by direct infusion (11) or via a sustained release mechanism (12) in order to achieve and maintain adequate tissue concentrations. Subsequently, studies by Vlodavsky et al. (13) demonstrated that basic FGF is sequestered by glycosaminoglycans in the extracellular matrix, the latter serving as a repository for the peptide. More recently, we found that the elimination half-life of pharmacologic doses of basic FGF was on the order of 60 min., not 2 min. as previously reported. These observations suggested to us that intermittent injection of large boluses of basic FGF could yield therapeutic concentrations in tissue, knowledge that vastly simplified our experimental models.

Our primary interest has been to foster collateral growth in patients with chronic myocardial ischemia. Accordingly, our animal models have involved the gradual occlusion of one or more coronary arteries, mimicking the pathophysiology of occlusive coronary artery disease in humans. Thus, we have implanted ameroid constrictors on the proximal left circumflex coronary artery (LCX) of dogs (Fig. 1). In the course of 10 - 20 days, these devices cause progressive arterial compression and ultimately, thrombotic occlusion. Dogs have a natural tendency to develop collaterals under these circumstances (infarcts are small and tend to be the exception rather than the rule), and the goal of our studies has been to promote the development of these collaterals.

In recent studies in which we assessed the effects of basic FGF and VEGF, the polypeptides (or placebo) were injected as a daily bolus directly into the LCX at a point just distal to the obstruction (3,4). Collateral blood flow to the LCX territory was quantified on a weekly basis during pharmacologically induced maximal coronary vasodilation. Both basic FGF (110 µg/d) and VEGF (45 µg/d) increased collateral flow by 40% after 4 wk. of treatment, and both peptides increased the number of blood vessels in the collateral-dependent myocardium. We also found a significant increase in cell proliferation in the collateral-dependent zone of basic FGF-treated dogs, supporting the suggestion that the increases in vessel number and myocardial perfusion were the result of an angiogenic mechanism.

Having determined that intracoronary administration of basic FGF and VEGF enhanced collateral development, we evaluated the effects of systemic basic FGF administration, and found that left atrial injection of basic FGF (1.74 mg/d for 4 wk) accelerated collateral development without major adverse effects. In a more ambitious study in which basic FGF was given systemically at the same dose for 5 or 9 wk (14), we found that treatment during the period of most pronounced ischemia (10 - 17 days after implantation of the constrictor) was important in enhancing collateral development, whereas treatment beyond this interval was not of additional benefit. We also found that the effects of basic FGF were sustained, persisting after withdrawal of treatment. More recently, therefore, we have limited the interval of basic FGF treatment to 7 days, and still obtained substantial increases in collateral blood flow (15).

We had largely discounted a potential role for growth factors in acute MI, because reperfusion of acutely ischemic myocardium must occur within 4 - 6 hours of coronary occlusion in order to avert infarction, whereas the angiogenic process governed by growth factors requires far longer to reestablish perfusion. Despite these theoretical concerns, intriguing studies by Yanagisawa-Miwa et al. (16) have demonstrated salutary effects of basic FGF on aspects of left ventricular function and infarct size after acute MI in dogs, and several groups have observed transcription of growth factor mRNA or growth factor bioactivity at various times after acute coronary occlusion in animals. These studies suggest a physiologic role for basic FGF in acute MI, both as a cardioprotective agent and as a mediator of infarct healing and remodeling.

To date, we have studied the effects of basic FGF in four independent studies in 87 dogs, and the results consistently demonstrate that the peptide stimulates coronary collateral development in this species. A major question, however, is whether these results can be extrapolated to humans with obstructive coronary artery disease. Tissue specificity is an important concern when basic FGF therapy for humans is being considered. As a nonspecific stimulator of proliferation in mesenchyme-derived cells, basic FGF has the potential to cause renal mesangial cell proliferation and myelophthisis with prolonged exposure to high doses, and this has been borne out in toxicology studies in animals. We are hopeful that such adverse effects can be avoided by limiting the dose and duration of treatment, a focus of current studies. The potential of basic FGF to accelerate tumor formation also needs to be considered. Obviously, the use of basic FGF in patients with known tumors would be contraindicated. Basic FGF does not have the ability to transform cells; however, it could potentially facilitate the growth of tumors in which an inadequate blood supply is the rate-limiting step. Another conceivable pitfall of angiogenic therapy relates to the potential of basic FGF to induce vascular smooth muscle cell proliferation, because neointimal smooth muscle cell hyperplasia is a fundamental component of atherosclerosis. Thus, basic FGF treatment could be a two-edged sword, enhancing collateral growth while accelerating atherosclerosis. We have preliminary data to suggest that this is not the case (15) and are planning additional studies to evaluate this issue further.

Clearly, the investigative area in which most work is needed is in elucidating the physiologic role of each growth factor and the intricacies of growth factor - growth factor and growth factor - receptor interactions. These factors may function through a complex cascade, in much the same way as the clotting factors do. Our current ignorance with respect to the growth factors is analogous to knowing the effect of placing thrombin on a bleeding wound but understanding nothing of the coagulation pathways. Nevertheless, we are cautiously optimistic about the potential clinical ramifications of these data and are currently planning phase I clinical trials to determine the pharmacokinetics and safety of basic FGF in humans. ■

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PLASMACYTOMAS IN BALB/C MICE

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Composition of Silicone Gels

Silicone gels are made by cross-linking liquid, linear silicone copolymer chains (4). The most commonly used linear silicone copolymers are methylhydrogenpolysiloxane and vinylmethylpolysiloxane:

The polymers are a mixture of molecules of different lengths. They include many low-molecular-weight chains and cyclic silicone intermediates that are side products during the purification of single strands of long polymers. These polymers are characterized by their viscosity, which reflects chain length; 1000 centiStokes DMPS has around 330 silicone atoms (pristane has only 15 linear carbons). To generate cross-linking sites, hydrogens or vinyl groups along the chains are covalently joined by adding the catalyst platinic chloride, which reduces the double bond of the vinyl group and links it covalently to the hydrogen on another chain. Platinum is known to be an immunologically active substance and appears to induce lymphocyte proliferation as well as allergic skin reactions (5). It is probably difficult to remove all of the platinum and many of the lower-molecular-weight silicone polymers from the gels, so we cannot rule out the possibility that some of the effects we observed were due to these materials. Also, cross-linking is never carried to completion, and as a result, the so-called silicone gels consist of a web of cross-linked silicones wrapped around liquid silicones. The incompletely cross-linked silicones are used for implants because they are soft, not hard and rubbery like completely crosslinked silicone. The incompletely cross-linked gels contain residual vinyl groups in linked polymer residues and, possibly, low-molecular-weight liquids as well, a potential source of highly reactive molecules.

Vinyl chloride (which is probably not present initially in the gel) has been shown to be epoxidized in cells to form DNA adducts with guanine (6), and vinyl acetate can be metabolized to acetaldehyde, which also cross-links DNA (7). We are now exploring whether the silicone gels can be broken down into low-molecular-weight, vinyl-containing molecules that can be taken into, and metabolized in B lymphocytes. Only fragmentary evidence so far suggests that silicone polymers can be biodegraded (8). Analyses of the silicone gels carried out by Xiaokui Zhang and Henry Fales of NHLBI show that low-molecular-weight linear and cyclic polymers with as few as four silicone units are present in the gels (3). The biological activity of these compounds, especially of the cyclic compounds octadimethyltetrasiloxane and vinylmethyltetrasiloxane, needs to be studied.

Genetic Predisposition of Plasmacytoma Induction in BALB/c Mice

A critical factor for the plasmacytomagenic process in mice is determined by genotype of inbred BALB/c strain of mice. This strain could be regarded as a "natural mutant" born with a predisposition to develop PCTs, but only when appropriately stimulated. Most other inbred strains that have been tested are resistant to developing PCTs after pristane or silicone-gel treatment. These strains carry PCT-resistance genes. Genetic analysis of first-generation backcross hybrids derived from suceptible BALB/c and resistant DBA/2 mice has been carried out by Beverly Mock of our laboratory (9). She has identified two PCT-susceptibility genes on mouse chromosome 4. Using a series of BALB/c.DBA/2 congenic strains constructed in our laboratory, we found 2 PCT-resistance genes, also on chromosome 4 (10). These susceptibility and resistance genes are probably alleles. Mice carrying PCT-resistance genes develop typical oil granulomas, and it is possible to find foci of proliferating atypical plasma cells in them; however, in most of these mice the number of foci is smaller than it is in susceptible mice.

Chromosomal Translocations that Activate the c-myc Protooncogene

The most important clue about the neoplastic phenotype in PCTs comes from cytogenetic studies carried out in collaboration with Francis Wiener at our laboratory (11). Over 95% of the PCTs induced by pristane or silicone carry chromosomal translocations that directly or indirectly involve the *c-myc* oncogene, such as the t(12;15) translocation, that deletes part of the *c-myc* gene and links it directly to an Ig heavy-chain switch-region gene. The *Sa* site is the preferential target among the seven switch sites occurring in 60% or more of the PCTs. Recently, Siegfried Janz and Jürgen Müller in our laboratory have developed a PCR assay for detecting *c-myc-Sa* illegitimate recombinations (12).

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STATUS OF INTRAMURAL MINORITY SCIENTISTS FINAL REPORT OF THE COMMITTEE

continued from page 1.

Results

A. Representation of underrepresented minorities on the intramural scientific staff of NIH.

As of Oct. 1, 1992, there were 1,148 tenured intramural scientists at NIH. Of these, 25, or 2.18%, were identified as underrepresented minorities; 0.70% were Black and 1.48% were Hispanic. There were no tenured Native American or Alaskan Native scientists. All eight tenured Black scientists were U.S. citizens when they came to NIH, whereas only 8 of 17 tenured Hispanic scientists were U.S. citizens when they came to NIH. Nine were educated abroad and came to NIH as visiting researchers. Thus, only 16 (8 Black and 8 Hispanic) scientists (or 1.39%) of the 1,148 tenured intramural investigators were U.S. citizen members of minority groups.

Of the 3,200 nontenured scientists at NIH on Oct. 1, 1992, 165, or 5.15% were identified as underrepresented minorities. Eighty-one (2.53%) were Black, 83 (2.59%) were Hispanic, and 1 (0.03%) was a Native American. Considering only U.S. citizens, there were 61 Blacks, 31 Hispanics, and 1 Native American among the 3,200 nontenured scientists, or 2.9 percent of the total.

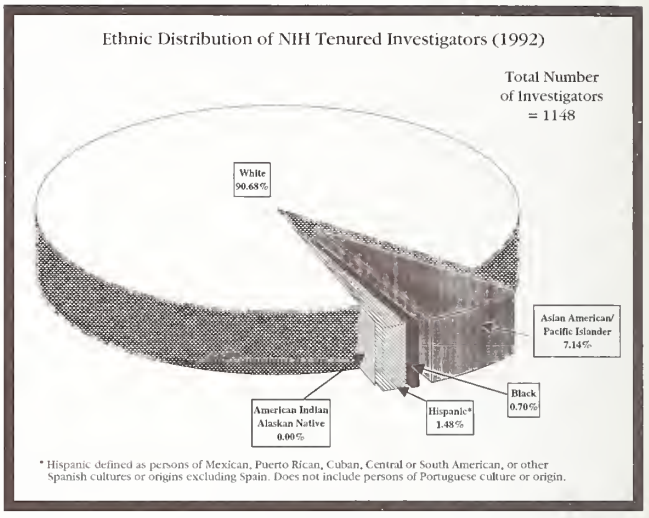
B. Recruitment and retention of underrepresented minorities.

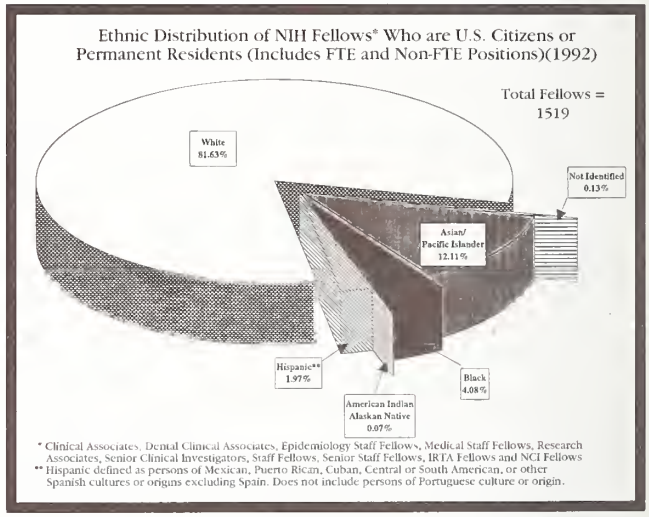
The programs established by the Office of Education and by the component institutes of NIH to recruit and retain minority scientists were reviewed by the committee. Efforts to recruit underrepresented minorities appear to be most effective when they are aimed at high school, college, and medical students. For example, 32% of high school summer interns at NIH in 1992 were underrepresented minorities, as were 22% of college undergraduate interns and 25% of medical students.

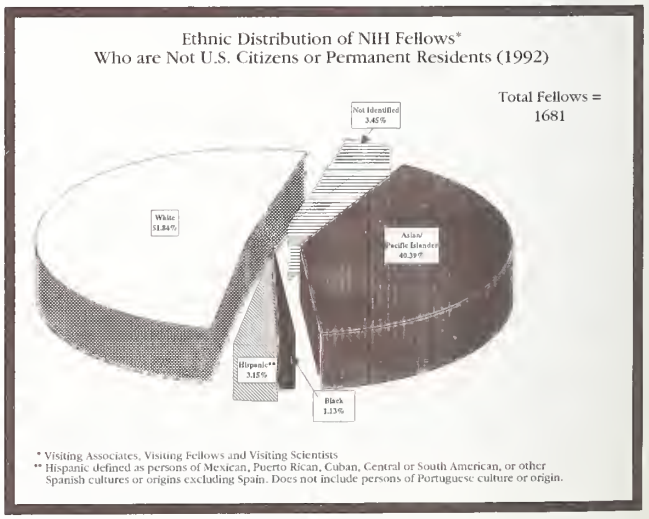
Minority targeted programs such as the Minority Access to Research Careers program of the National Institute of General Medical Sciences, and other institute-initiated programs accounted for 43% of all minorities recruited to summer internships at NIH, whereas the much larger general summer internship programs contributed 57%. The percentage of minority students in the summer internship group varied considerably among the institutes; for those with greater than 25 students, the percentage ranged from 7% (National Eye Institute) to 39% (National Institute of Diabetes and Digestive and Kidney Diseases).

At the postdoctoral level, there are few targeted minority-recruitment initiatives. Although many positions are nationally advertised and minority applications are encouraged, there is no NIH-wide effort to target and reach minority M.D. or Ph.D. scientists.

Both tenured and nontenured scientists felt that effective recruitment efforts for underrepresented minority scientists have not been implemented. Nontenured scientists expressed concern that there were few role models among tenured scientists, and the majority of the nontenured minority scientist interviewed noted serious deficiencies in their mentoring relationships with their supervisors. Many untenured minority scientists experienced feelings of isolation from peers and noted an absence of an effective network of minority scientists on the NIH campus. Few had received explicit information regarding NIH tenure procedures, and few had a clear understanding of their own status with respect to tenure-track and tenure potential. Although most underrepresented minority scientists felt that NIH offered significant opportunities for training and productive career development, many also felt that minority scientists were not readily accepted as peers and at times, experienced overt discrimination. All expressed the opinion that explicit training and periodic evaluation of supervisors with respect to racial and gender discrimination, mentoring, and recruitment of minorities should be required.







Recommendations

A. General

After analyzing the data gathered in interviews and surveys, the committee recommends the following:

- Create a full-time position for a Director of Minority Science Faculty Development within the Office of the Deputy

Director for Intramural Research (DDIR). This person would be responsible for reviewing, monitoring, and initiating efforts to recruit and retain minority scientists and would be specifically charged with overseeing the implementation of this report. The incumbent would be assisted by an Advisory Committee on Minority Science Faculty Development.

- Maintain data on the racial and ethnic origins of all intramural scientists through the NIH Division of Personnel Management.

- Require each ICD Scientific Director to provide a yearly report to the Office of the DDIR on the ICD'S efforts to increase participation of minorities in its intramural program.

- Require each intramural site-visit team to specifically address the representation of minority scientists in the laboratory or branch under review and to evaluate programs and plans to increase minority scientists representation, conversion to tenure, promotion, retention, and research support. The quality of mentorship in the laboratory or branch should be a necessary factor in the site-visit evaluation. The site-visit report should be provided to the Office of the DDIR.

- Require all supervisors in the IRP-NIH, as part of their yearly performance review under the EEO critical element, to document their efforts to increase the recruitment of minority scientists for open positions and to document efforts to retain minority scientists in their area of responsibility. Efforts are to be documented in a detailed fashion.

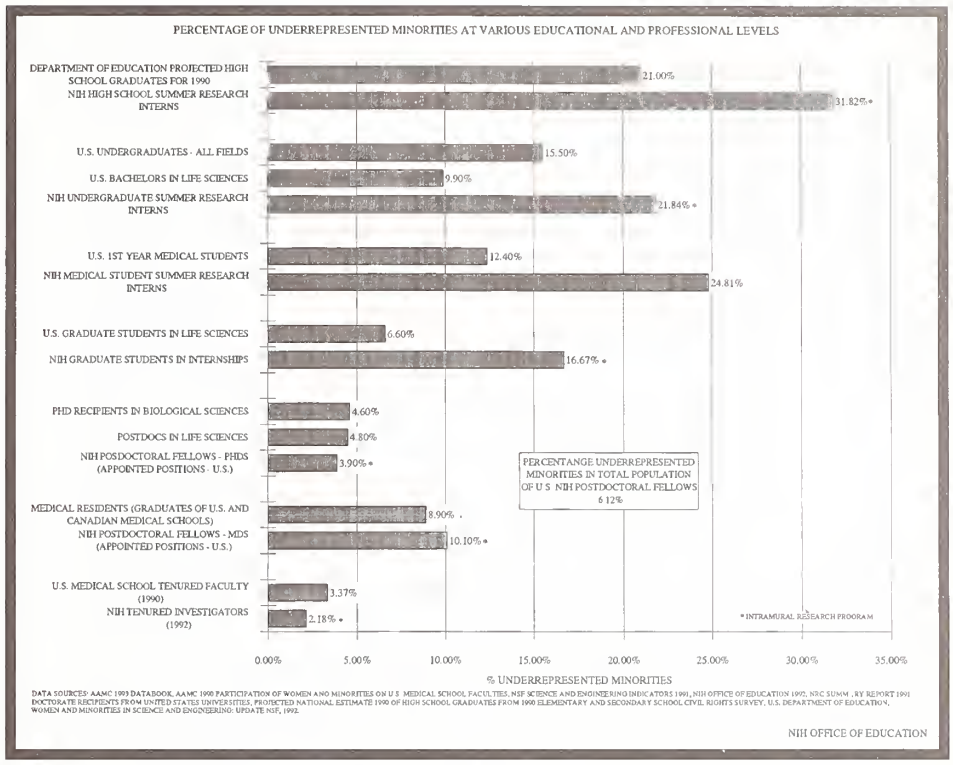
- Consider applicants for postdoctoral fellowship programs that encumber full-time equivalents at NIH under the NIH Federal Equal Opportunity Recruitment Program (FEORP) policy.

- Require annual training for all intramural scientists in areas concerning cultural diversity, race discrimination, attitudes and behaviors, prevention of sexual harassment, and mentoring.

- Implement a program for loan forgiveness for underrepresented minority scientists in IRP-NIH, consistent with the 1993 legislative reauthorization of NIH.

B. Recruitment

Establish a position for recruitment of minority scientists in the Office of Education, NIH. The recruitment office would take responsibility for establishing new programs and amending existing programs to increase recruitment of minorities to internship programs, predoctoral and postdoctoral research positions, and tenured faculty positions NIH-wide.



The recruiter's activities would include programs to

1. Maintain a database on all underrepresented minority students and postdoctoral scientists who work at NIH to track their career progress.

2. Develop a mailing list and database of minority predoctoral and postdoctoral scientists nationwide and regularly provide them with information on positions available at NIH.

3. Establish a network for underrepresented minority scientists on the NIH campus as a way to increase support and communication for nontenured scientists.

4. Develop and implement a program, with designated funding, to foster faculty exchange and recruitment of students for internships from historically Black colleges and universities, from predominantly Hispanic colleges and universities, and from institutions that have significant numbers of Native American students.

5. Develop programs for improving the mentoring of postdoctoral scientists in IRP-NIH.

In addition, encourage and recruit scientists from African countries to participate in the Fogarty Visiting Program.

In considering alternatives for new recruitment efforts, the Office of Education should be given adequate resources to implement NIH-wide those existing programs (particularly those aimed at summer internships and early postdoctoral training) that have successfully recruited minority scientists to selected NIH institutes.

C. Retention and Promotion

The ICDs should:

1. Advertise and open up for competition all tenure-track positions under FEORP guidelines.

2. Ensure representation of underrepresented minorities on promotion and tenure-review panels, site-visit teams, and boards of scientific counselors.

3. Provide orientation to all postdoctoral fellows regarding NIH tenure-track policies and tenure processes at the time the fellows enter IRP-NIH.

4. Ensure the participation of underrepresented minorities as speakers and participants in scientific programs organized and/or sponsored by the ICD.

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Hot Methods Clinic
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Termination of the ligation reaction is achieved by diluting the reaction mixture 1:10 with sterile, distilled water. In most cases, 1-5% of the diluted mixture is used in subsequent amplification reactions.

cDNA Amplification

Regardless of the strategy employed, "hot start" PCR using 50 µL reaction volumes is recommended. Use 10 pmol each of the anchor primer and the G2 or the G3 primer with cDNAs modified by the anchor oligomer ligation method; use 10 pmol of the tailed anchor primer and 25 - 100 pmol each of the anchor primer and the G2 or G3 primer with cDNAs modified by the tailing method. (Note: The 5' RACE System kit does not use a mixture of tailed and non-tailed anchor primers as the upstream oligomers; instead, only an anchor primer with a modified homopolymeric tail is used.) The following cycling parameters should yield good results:

94°C, 45 s.
55°C, 45 s.
72°C, 2 min.
for 35 cycles.

Troubleshooting Tips

1. Intact mRNA is absolutely essential for the success of 5' RACE. If possible, a northern blot of the starting RNA should be made to verify its integrity.

2. The cDNA primer should be designed to anneal at least 200 bases downstream from the 5' end of the known mRNA sequence. The resulting cDNA will, therefore, contain a stretch of known sequence. Amplification reactions using oligomers complementary to the ends of this region will allow confirmation that the correct target sequence has been copied. A necessary control here is the amplification of mock cDNA (i.e., from a reaction in which RT was omitted); this will establish that the final product was generated from mRNA and not from contaminating genomic DNA.

3. If cDNA yields in the first step are low, purification using a silica matrix may be risky. In this case, purify the cDNA by size-exclusion chromatography or by gel electrophoresis. If the cDNA is purified electrophoretically, it will have to be labeled to locate its position in a gel.

4. Incomplete cDNA synthesis, nonspecific priming by the oligomers used in cDNA synthesis and PCR, and PCR artifacts such as primer-dimer formation may yield complex 5' RACE products. Therefore, it may be necessary to try several different gene-specific oligomers and to vary the amplification protocol to achieve a good result. Changes in the amplification conditions may include altering the oligomer annealing temperature, using a different $MgCl_2$ concentration, using a different thermostable DNA polymerase, and adding 10% dimethyl sulfoxide (DMSO) to the PCR mixtures. If no specific product is observed after the first round of PCR, prepare a Southern blot with some of the amplified material to see whether a small amount of the desired species is present (use the known cDNA sequence as a probe). If the desired product is present, re-amplification with another nested, gene-specific primer may improve the yield.

5. Use of nested, gene-specific primers enhances the specificity of the amplification reactions. However, control reactions using only 5' or 3' primers are recommended to establish bilateral priming of the PCR products.

6. The oligomers used in the amplification reactions should contain restriction enzyme recognition sequences at their 5' termini to facilitate cloning of the products; the tailed anchor primer is synthesized with a homopolymeric tail of approximately 15 residues at its 3' end.

5' RACE Contacts

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Plasmacytomas in BALB/c Mice
continued from page 19.

We have been able with this PCR methodology to consistently detect illegitimate recombinations of *c-myc* and *Sa* in cells from pristane oil granulomas 30 days after the injection of pristane. Thus, the *c-myc-Sa* recombination is a very early event and is potentially the initiating mutation that leads to PCT development. We postulate that some of the cells bearing these *c-myc-Sa* recombinations are the clonal precursors of the PCTs.

Hypothetical Scheme of PCT Development

A speculative scenario that fits most of the facts is that BALB/c mice, for genetic reasons, have a high predilection to develop illegitimate recombinations between *c-myc* and Ig loci. This oncogenic mutation results in deregulation of *c-myc* transcription such that the *c-myc* gene cannot be turned off. Unregulated transcription is probably a critical change because *c-myc* is shut down when cells exit from the cell cycle. Plasma cells are thought to be an end stage of B-cell development and usually cease dividing. Cessation of *c-myc* transcription may drive the cell into a postmitotic state. Rearrangement of the *c-myc* gene in t(12;15) removes the normal negative control sites that govern transcription. The mutant plasma cells have a continuous supply of *c-myc* protein, which may make it difficult for the cell to exit from the cell cycle, yielding the paradoxical phenotype of a mitotically active, terminally differentiated plasma cell. Cells with these illegitimate recombinations are probably eliminated or are not a cause of tumor formation, but they survive in the chronic inflammatory tissue, possibly because of the high concentrations of various growth factors (13,14). During this survival period, late-acting progressor genes may play a crucial role in rescuing the cells from cell death and other forms of elimination. This allows further time for changes that permit the cells to adapt and proliferate without control.

Implications

What does this imply for women with silicone-gel implants? Leaky breast implants in humans release silicone materials into connective tissues, where it induces a granulomatous tissue quite similar histologically to that in the mouse peritoneum (15,16). Silicone gels are known to be immunological adjuvants in experimental

animals (17). Leaky implants have been reported to occur in around 0.2% to 1.1% of cases (18), but the Council on Scientific Affairs of the American Medical Association suspects the incidence of leakage is higher. In unconfirmed studies, leaky implants have been reported to raise the levels of IgG molecules that react with silicone-like materials (19). Because silicone gels do have immunostimulatory properties, it may be useful to evaluate clinically how individuals respond by obtaining a quantitative analysis of the various classes of immunoglobulins and a serum protein electrophoresis, possibly using immunofixation.

It is reassuring, though, that PCT development in mice is highly dependent on a rare and unique genetic constitution — one that may never occur in humans. In mice, only the peritoneal granulomatous tissue is important in this process, and this tissue site is probably not involved in granuloma formation in humans. Also, local PCTs have not been reported as a complication of an implant. Finally, mouse plasmacytomagenesis is dependent on *c-myc* activating chromosomal translocations. Although homologous translocations such as t(8;14) occur in Burkitt's lymphomas in humans, they very rarely occur in human plasma cell tumors. ■

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Recently Tenured

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eases. Some of these inhibitors have already been shown to have therapeutic value in preventing scrapie in animals. We hope that these compounds may be effective as drugs not only for TSEs but also for amyloidoses of greater clinical significance in humans, such as Alzheimer's disease.

One of the major difficulties in studying the underlying basis for TSE disease was the fact that no one had been able to convert the normal PrP to PrP-res in anything simpler than a scrapie-infected cell. In collaboration with Peter Lansbury's lab at the Massachusetts Institute of Technology, we recently overcame this problem by establishing a defined, cell-free reaction mixture that supports the formation of PrP-res. This discovery provided the first direct evidence that PrP-res formation results simply from an interaction of normal PrP with preexisting PrP-res. With this experimental system, we now have a unique opportunity to study the chemical details of this process, which is central to the TSE diseases, and to analyze the nature of the infectious agent that instigates this disease process in the host. ■

Status of Intramural Minority Scientists
Final Report of the Committee

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D. Additional Concerns

1. Among the concerns voiced by minority scientists were the following. The health problems of minorities should be addressed by the IRP-NIH. The ICDs should be committed to ensuring proportional representation of minority patients in NIH's clinical programs. The committee strongly encourages the Office of Research on Minority Health to involve intramural scientists in the planning of regular NIH conferences on minority health. NIH should organize an initial national conference addressing the problem of underrepresentation of minority scientists in biomedical research.

2. The particular problems experienced by minority women scientists should be addressed by NIH's Women Scientist Advisors and the Committee on the Status of Intramural Minority Scientists. ■

Eric Francoeur, the 1994-95 Stetten Memorial Fellow, is currently doing research on the design and use of mechanical molecular models, such as the CPK space-filling models or the Kendrew skeletal models. He would be interested to hear from NIH scientists who have used or are still using such models in their research. He can be reached by mail (Building 31, room 2B09), phone (496-6610), or E-mail (stetten@helix.nih.gov). ■

FAX-BACK

In this issue we ask for feedback in five areas: Minority Task Force report; suggestions for our Hot Methods Clinic; what constitutes a well-run lab; NIH fellows' problems with computer and network services; and problems related to the reasonable pricing clause. **Fax your responses to 402-4303** or mail it to us at Building 1, Room 134.

In Future Issues. . .

- OAR's New Director
- Extramural NIH, Part III: Mentorship at NIH
- IRP Scientsts Rate the Research Journals
- Hot Methods Clinic: Phage Display and Epitope Libraries

1) What comments do you have on the Minority Task Force report, and what suggestions would you make as NIH moves to implement the report's recommendations?

2) Do you have any tips or comments on 5' RACE, featured in this issue's Hot Methods Clinic? Do you have any tips for our next Hot Methods Clinic feature: Phage Display and Epitope Libraries. What techniques would you like to see covered in future issues?

3) What are your worst experiences with crowded lab conditions; what are the ingredients of a well-run NIH lab? Which is the best-run lab on campus?

4) Specifically for NIH fellows: Have you had difficulties gaining access to computer and network services at NIH? If so, what were your problems?

5) NIH is reviewing technology transfer policies. In attempting to establish CRADAs or other collaborations with private companies, have you experienced any problems attributable to the reasonable pricing clause? Please provide details (on a separate sheet, if necessary).

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