CCR frontiers IN SCIENCE

May 2003, Volume 2 Published by the Center for Cancer Research, National Cancer Institute

Contents

Structural Biology 1
Cyanovirin-N Hits the "Sweet Spot" of HIV

Cancer and Cell Biology . . .3
Positional Cloning of the Birt-Hogg-Dubé Disease Gene from a Region of Unique Genome Architecture

Chromatin Dynamics: Competition Between Nucleosome-binding Proteins for Chromatin-binding Sites

Immunology 6
Autoimmune Antigens Bring Together Innate and Adaptive Immune Responses by Chemoattracting Immature Dendritic Cells

Biotechnology Resources . . .7
Cell-free Protein Synthesis—An Old Dog with New Tricks

Clinical Research 9
Reaching Out to Special Populations: CSSC Boosts Referrals to NCI Clinical Studies

From the Director 10
The Medical Oncology Research Unit: A Cornerstone of Excellence in Clinical Research and Patient Care

On the Tenure Track 12
Dr. Brad St. Croix

Administrative Links 12

NATIONAL CANCER INSTITUTE
NATIONAL INSTITUTES OF HEALTH
DEPARTMENT OF HEALTH AND HUMAN SERVICES

■ STRUCTURAL BIOLOGY

Cyanovirin-N Hits the "Sweet Spot" of HIV

Botos I, O'Keefe BR, Shenoy SR, Cartner LK, Ratner DM, Seeberger PH, Boyd MR, and Wlodawer A. Structures of the complexes of a potent anti-HIV protein cyanovirin-N and high-mannose oligosaccharides. *J Biol Chem* 277: 34336-42, 2002.

Of the more than 30 million people infected with human immunodeficiency virus (HIV) before 1997, 75 to 85 percent acquired the virus through heterosexual contact, making acquired immunodeficiency syndrome (AIDS) a continuing threat to the general population. The development of a vaccine active against multiple HIV clades is complicated by the high mutation rate of the virus. Another route to prevention is the development of anti-HIV virucides for topical or *ex vivo* use. A unique natural product with anti-HIV properties was discovered while screening for new antiviral agents. This protein, originally isolated from cultures of the cyanobacterium (blue-green algae) *Nostoc ellipsosporum*, was named cyanovirin-N (CV-N).

In nanomolar concentrations, CV-N potently inactivates HIV-1, HIV-2, simian immunodeficiency virus (SIV), and feline immunodeficiency virus (FIV). It acts at the level of the virus, not the target cell, to abort the infection process by preventing essential interactions between the heavily glycosylated HIV-1 envelope glycoprotein gp120 and the target cell receptors. Since the antiviral activity of CV-N results from this protein's binding to carbohydrates, understanding the structural basis of such interactions is important for the potential development of CV-N as an anti-AIDS agent.

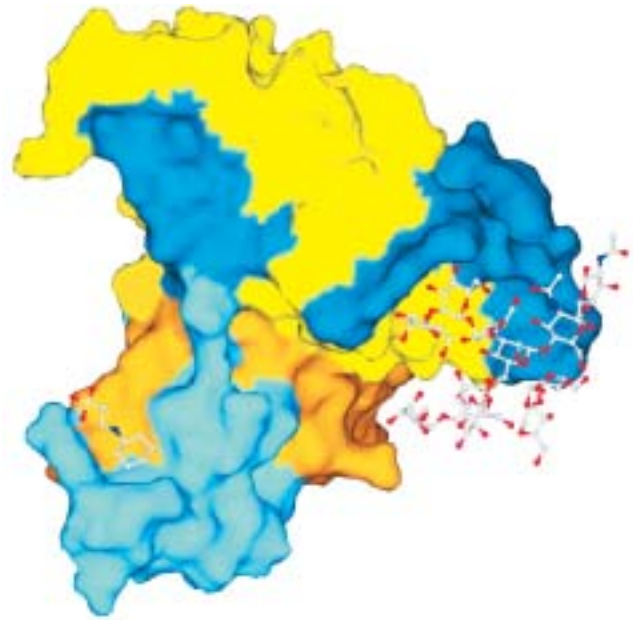

Figure 1. Crystal structure of the domain-swapped CV-N dimer bound to Man-9. Presented here is the molecular surface with the primary and secondary oligosaccharide-binding sites, with a 2-(cyclohexylamino)ethanesulfonic acid (CHES) buffer molecule bound to the former (*left*) and Man-9 to the latter (*right*). Domains A (dark blue) and B (light blue) from the first molecule and domains A' (orange) and B' (yellow) from the second molecule are shown. AB' (dark blue and yellow) and A'B (light blue and orange) are the pseudo-monomers.

The structure of CV-N has been studied by both crystallography and nuclear magnetic resonance (NMR). Monomeric CV-N (11 kDa) has two similar domains, A and B, linked by a hinge region. A change in torsion angles in the hinge region separates the domains into an extended form in which they do not contact each other.

A domain-swapped dimer, observed in all crystal structures, is formed by two such extended monomers in which domain A comes in contact with domain B' from another extended monomer. The overall structure of each pseudo-monomer (AB' and A'B; Figure 1) is virtually identical to the compact monomer except for the hinge residues. Two different crystal forms of CV-N, high pH and low pH, have been described. The relative orientation of the pseudo-monomers is different between the structures at high and low pH. We performed our sugar binding studies of CV-N with the high-pH crystal form.

Since the primary sugar-binding site [of cyanovirin-N] is close to the hinge region, the position of the hinge and the relative orientation of the domains directly affect the shape of the primary sugar-binding site.

We used two branched high-mannose oligosaccharides in this study. Oligomannose-9 (Man-9) was derived from natural sources and its structure corresponds to that of a gp120 oligosaccharide. The second oligosaccharide is a synthetic hexamannoside with the mannoses linked similarly to the core structure of Man-9. CV-N binds to Man-9 with nanomolar affinity and to the synthetic hexamannoside with low micromolar affinity. A CV-N monomer has two distinct sugar-binding sites: a high-affinity, primary site and a low-affinity, secondary site. NMR and isothermal titration calorimetry experiments have shown that the binding sites exhibit different affinities for the high-mannose oligosaccharides. A domain-swapped dimer has four sugar-binding sites: two primary sites near the hinge region, and two secondary sites on the opposite sides of the dimer not influenced by the conformation of the hinge region. In the domain-swapped dimer the sugar-binding sites are formed by intertwined loops of residues on the pseudo-monomer.

The primary sugar-binding site consists of a deep pocket close to the hinge region. A tightly bound 2-(cyclohexylamino)ethanesulfonic acid (CHES) molecule from the crystallization solution was found bound to this site in our structures. Since the primary sugar-binding site is close to the hinge region, the position of the hinge and the relative orientation of the domains directly affect the shape of the primary sugar-binding site. In all domain-swapped crystal structures some of the essential protein—oligosaccharide hydrogen bonds are not favorable.

The secondary sugar-binding site, unaffected by the hinge region, has the same conformation in both the monomeric and domain-swapped dimeric CV-N. The protein-binding interface, as revealed by the structure, is formed by three mannose rings in Man-9 and by two mannose rings in hexamannoside. The additional binding affinity of CV-N for Man-9 compared with hexamannoside is the result of the additional binding energy derived from the third mannose ring. Man-8 and Man-9 oligosaccharides are rare and exist only on short-lived glycoproteins in humans, but are much more prevalent on viral glycoproteins. As many as 11 high-mannose oligosaccharides can exist on HIV gp120, 5 or 6 of these generally in the form of Man-8 or Man-9.

The ability of CV-N to target virus-associated oligosaccharides with high affinity, while binding mammalian oligosaccharides (e.g., Man-6) with comparably low affinity, is the basis for the potential utility of this agent as a specific anti-HIV microbicide.

■ **Istvan Botos, PhD**
Post-doctoral Fellow
■ **Alexander Wlodawer, PhD**
Chief, Macromolecular Crystallography Laboratory
NCI-Frederick, Bldg. 536/Rm. 5
Tel: 301-846-5036
Fax: 301-846-6128
wlodawer@ncifcrf.gov

Positional Cloning of the Birt-Hogg-Dubé Disease Gene from a Region of Unique Genome Architecture

Nickerson ML, Warren MB, Toro JR, Matrosova V, Glenn G, Turner ML, Duray P, Merino M, Choyke P, Pavlovich CP, Sharma N, Walther M, Munroe D, Hill R, Maher E, Greenberg C, Lerman MI, Linehan WM, Zbar B, and Schmidt LS. Mutations in a novel gene lead to kidney tumors, lung wall defects, and benign tumors of the hair follicle in patients with the Birt-Hogg-Dubé syndrome. *Cancer Cell* 2: 157-64, 2002.

The genetic causes of epithelial tumors of the kidney have been elucidated by study of families with multiple members affected with renal cancer (Zbar B. *Semin Cancer Biol* 10: 313-8, 2000). So far, this approach has led to identification of renal cancer-causing mutations in the *von Hippel-Lindau*, *c-Met*, and *Fumarate Hydratase* genes as well as cancer-causing translocations involving chromosome 3. Recently, NCI scientists in the Laboratory of Immunobiology have discovered a novel gene associated with renal oncocytoma and chromophobe renal carcinoma. This work was conducted in collaboration with scientists from NCI's Urologic Oncology, Genetic Epidemiology, and Dermatology Branches and the Pathology and Diagnostic Radiology Laboratories. The observation of tumors of the hair follicle known as fibrofolliculomas in Birt-Hogg-Dubé (BHD) syndrome allowed effective recruitment of renal cancer families and accurate identification of affected family members. Detailed study of these families revealed an additional, stronger association between renal cancer and hair follicle tumors with lung cysts and lung collapse (spontaneous pneumothorax) (Zbar B et al. *Cancer Epidemiol Biomarkers Prev* 11: 393-400, 2002).

Using 185 polymorphic microsatellite markers, a genome-wide scan was accomplished by genotyping 40 members of the Canadian family originally described by Drs. Birt, Hogg, and Dubé (Schmidt LS et al. *Am J Hum Genet* 69: 876-82, 2001). This effort examined 19 autosomal chromosomes at a 10- to 15-cM resolution. Linkage was obtained on chromosome 17 in the 17p11.1–p12 pericentromeric region. The initial 8.5-cM region containing BHD candidate genes showed a maximal two-point LOD score of 4.98 at $\Theta = 0$ for marker D17S740. Subsequently, eight additional families were genotyped with six linked markers and produced a LOD score of 16 at $\Theta = 0$ for marker D17S2196. Critical recombinants in these families further reduced the BHD gene candidate region to less than 4 cM. Approximately 30 additional microsatellite markers were developed and, along with single-nucleotide polymorphisms, were used to characterize haplotypes associated with the disease phenotype in additional BHD families. This work identified a 700-kb region of 17p11.2 as being nonrecombinant for BHD (Nickerson ML et al. *Cancer Cell* 2: 157-64, 2002).

The BHD gene project was one of the first positional cloning successes that used comparative analysis of genome browsers to precisely locate microsatellite markers and candidate genes in the draft human genome sequence.

The timing and progress of the BHD gene hunt closely mirrored the Human Genome Project that sequenced and assembled the human genome. The BHD gene project was one of the first positional cloning successes that used comparative analysis of genome browsers to precisely locate microsatellite markers and candidate genes in the draft human genome sequence. Genome browsers analyzed included University of California–Santa Cruz, National Center for Biotechnology Information, Ensembl, Celera, and Doubletwist. Locations of draft sequence contigs were confirmed by three-color fluorescent *in situ* hybridization, PCR of overlapping BAC clone markers, and recombination mapping.

Development of a correctly oriented, final BAC contig was hindered by the presence of three low-copy repeats known as SMS-Reps. The proximal and distal SMS-Reps flank a roughly 4-Mb region that is deleted in Smith-Magenis syndrome (SMS). The repeats are large (approximately 200 kb), are highly homologous (over 98% similarity), and participate in nonallelic homologous recombination (unequal crossing over) to cause deletions in SMS patients (Park S-S et al. *Genome Res* 12: 729-38, 2002). The distal SMS-Rep was included in the BHD critical region and contained approximately 14 genes (Park S-S et al. *Genome Res* 12: 729-38, 2002). These genes were ruled out as candidates causing BHD on the basis of comparative genomics using rat and dog genomic data. The Nihon rat model of renal carcinoma (Hino O et al. *Jpn J Cancer Res* 92: 1147-9, 2001) and canine renal cystadenocarcinoma and nodular dermatofibrosis diseases (Jonasdottir TJ et al. *Proc Natl Acad Sci U S A* 97: 4132-7, 2000) are very similar to human BHD and link to genomic loci syntenic to and inclusive of the human BHD locus on 17p11.2. Because SMS-Reps are found only in higher primates, not in rat and dog, genes in the distal SMS-Rep were not considered *BHD* candidates. Researchers studying these BHD-like diseases in rat and dog are now analyzing their respective BHD gene homologs for disease-associated mutations.

The *BHD* gene lies about 250 kb from the distal SMS-Rep between the distal and middle repeats (Figure 1). Two overlapping transcripts were sequenced by

NIH's Mammalian Gene Collection group and were released by Genbank in October 2001. These transcripts were included in the December 2001 release of the University of California–Santa Cruz genome browser and were found to contain protein-altering sequence changes that cosegregated with BHD disease. Probes synthesized from coding sequence from either transcript revealed an approximately 3.8-kb transcript when hybridized to Northern blots. The normal cDNA was subsequently isolated. All mutations have been found to truncate the protein folliculin, named for the hair follicle hamartomas produced in BHD.

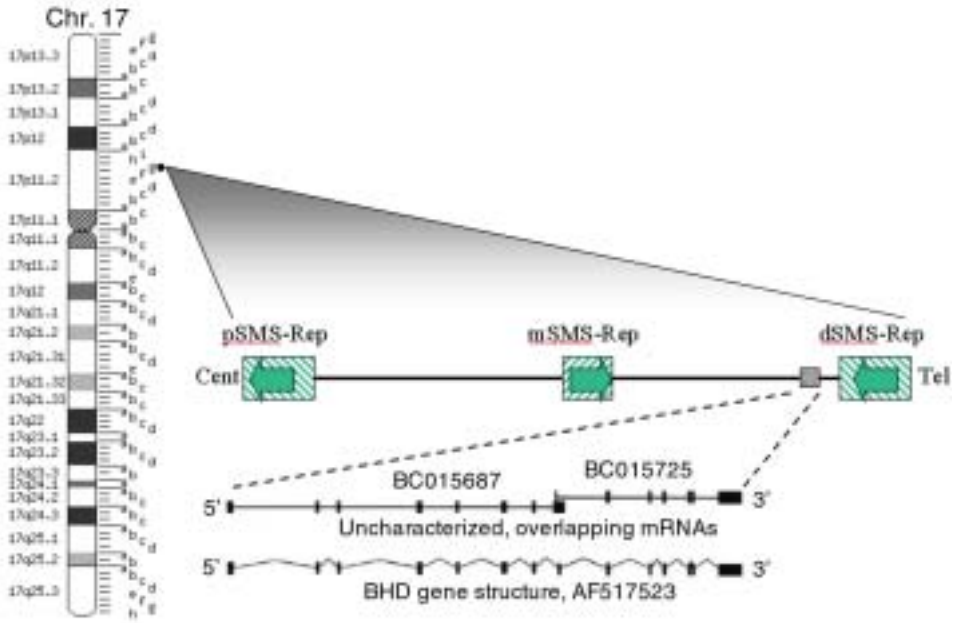

Figure 1. Location of the Birt-Hogg-Dubé (BHD) disease gene on chromosome 17. Green arrows indicate candidate genes and expressed sequence tag clusters examined for mutations by 96-well format sequencing on an ABI 3700 and denaturing high-performance liquid chromatography. The two overlapping transcripts (BC015687 and BC015725) were sequenced by NIH's Mammalian Gene Collection group. The normal cDNA (AF517523) was subsequently isolated. Cent, centromere; Tel, telomere; pSMS-Rep, mSMS-Rep, and dSMS-Rep, the proximal, middle, and distal low-copy repeats deleted in Smith-Magenis syndrome.

A mutation hot spot of eight cytosine nucleotides was altered by either an insertion or a deletion of a single cytosine in 44 percent of the BHD families tested to date. No related human gene homologs or Pfam domains were identified among known proteins, but folliculin is highly conserved across species and single-copy orthologs were identified in mouse, *Drosophila*, and *Caenorhabditis elegans*.

Studies are currently in progress to determine the mechanism whereby folliculin influences tumorigenesis. BHD patients are predisposed to develop different histologic types of renal cancer, including renal oncocytoma and chromophobe renal carcinoma. Possibly, the *BHD* gene may act as a developmental regulator in a progenitor cell that is switched to uncontrolled growth by mutant folliculin before the decision is made to specify the distinct type of renal tumor. It will be exciting to see how the renal, lung, and skin findings interrelate to elucidate the biological function of this new cancer gene.

- **Michael L. Nickerson, PhD**
  Biologist
- **Berton Zbar, MD**
  Chief, Laboratory of Immunobiology
  NCI-Frederick, Bldg. 560/Rm. 12-69
  Tel: 301-846-5857
  Fax: 301-846-6145
  nickersm@ncifcrf.gov

Chromatin Dynamics: Competition Between Nucleosome-binding Proteins for Chromatin-binding Sites

Catez F, Brown DT, Misteli T, and Bustin M. Competition between histone H1 and HMGN proteins for chromatin binding sites. *EMBO Rep* 3: 760-6, 2002.

The chromatin fiber is a dynamic structure in a constant state of flux. Global changes in the compaction of the chromatin fiber are easily observable as the cell progresses during the various stages of the cell cycle and the interphase chromatin condenses into mitotic chromosomes. Alterations in local chromatin structure can be observed during changes in the expression of specific genes or in response to DNA damage. Even the structure of the nucleosome, the building block of the chromatin fiber, is dynamic because it is continuously destabilized by multiprotein complexes that transiently disrupt the interactions between histones and DNA. The dynamic, malleable properties of the nucleosome and of the chromatin fiber facilitate the orderly progression of preprogrammed cellular processes and enable the cell to mount proper responses to changing environmental stimuli.

The dynamic nature of the chromatin fiber reflects the action of numerous regulatory factors that modify the histones and DNA and remodel the structure of the nucleosome. In part, access of these factors to their targets is modulated by nuclear proteins such as histone H1 and high-mobility group N (HMGN) proteins. These nuclear proteins interact with the periphery of the nucleosome and induce chromatin architectures that either impede or promote the enzymatic action of chromatin-modifying activities. Histone H1 stabilizes compact chromatin

structures, impedes the action of transcription activators, and reduces DNA accessibility. HMGN proteins reduce the compaction of the chromatin fiber, promote access to nucleosomes, and usually stimulate transcription. *In vitro* studies have demonstrated that HMGN proteins can alleviate histone H1-induced transcription repression and that the nucleosome-binding sites of histone H1 partially overlap with those of HMGN proteins. In living cells, the organization of both histone H1 and HMGN proteins is dynamic and their interactions with specific sites in chromatin are transient. These and additional observations raise the possibility that an interplay between histone H1 and HMGN proteins on the surface of the nucleosome may affect the dynamics of the local chromatin structure.

With Tom Misteli, PhD, at CCR we tested whether HMGN proteins affect the interaction of histone H1 with chromatin in living cells. Dr. Misteli used fluorescence recovery after photobleaching (FRAP) to demonstrate that most nuclear proteins constantly move throughout the nucleus. In the FRAP technique, a small area in a nucleus expressing a fluorescent protein is irreversibly photobleached and the rate at which the fluorescent signal in the photobleached area recovers is quantified. The rate of signal recovery is indicative of the rate at which unbleached molecules from the vicinity enter the photobleached area and exchange with the photobleached protein molecules. In turn, this rate is directly proportional to the rate at which a molecule moves throughout the nucleus and inversely proportional to the time that a molecule resides at its immobile binding site (in the case of histone H1 and HMGN proteins, chromatin). The binding of histone H1 to chromatin is significantly stronger than the binding of HMGN proteins to chromatin. Therefore, the chromatin residence time of histone H1 is longer: the apparent intranuclear mobility of histone H1 is almost 10 times slower than that of HMGN proteins.

We reasoned that if HMGN proteins and histone H1 compete for the same chromatin-binding sites, an excess of HMGN proteins would decrease the chromatin residence time of histone H1. We microinjected HMGN proteins into the cytoplasm of cells expressing fluorescent histone H1 and, using FRAP, we compared the intranuclear mobility of the fluorescent histone H1 in the injected cells with that of non-injected cells. The presence of exogenous HMGN proteins reduced the residence time of histone H1 on chromatin; that is, histone H1 moved faster in the nucleus. HMGN protein point mutants that do not bind to chromatin did not affect the mobility of histone H1, an indication that the two proteins compete for chromatin-binding sites. Indeed, treatment of cells with compounds that prevent the binding of HMGN proteins to chromatin or strengthen the binding of histone H1 to nucleosomes abolished the ability of HMGN proteins to compete with histone H1.

We previously noted that the binding sites of HMGN proteins on the nucleosome overlap with those of histone H1. We also reported that HMGN proteins alleviate histone H1-induced transcription repression. Taken together, these findings suggest that a dynamic interplay between HMGN proteins and histone H1 on the surface of the nucleosome may affect the local chromatin structure. High local concentrations of HMGN proteins may temporarily weaken the interaction of histone H1 with chromatin and decrease the compactness of the local chromatin fiber. In the decompacted regions, the nucleosomal DNA would be more accessible to regulatory factors. We have already published that in most nuclei HMGN proteins are concentrated in discrete foci and that these foci, representing high local concentrations of the protein, colocalize with nascent transcripts (Hock R et al. *EMBO J* 17: 6992-7001, 1998).

Our experimental approach is applicable to studies on many of the nuclear proteins that, in a fashion analogous to HMGN proteins, move rapidly throughout the nucleus and affect the structure and function of chromatin. The transient and competitive interactions of nuclear binding proteins with their chromatin targets impart the structural resilience necessary for the orderly progression of global and local changes in chromatin structure.

- **Frédéric Catez, PhD**
  Visiting Fellow
- **Michael Bustin, PhD**
  Principal Investigator
  Laboratory of Metabolism
  NCI-Bethesda, Bldg. 37/Rm. 2D-21
  Tel: 301-496-5234
  Fax: 301-496-8419
  bustin@helix.nih.gov

Autoimmune Antigens Bring Together Innate and Adaptive Immune Responses by Chemoattracting Immature Dendritic Cells

Howard OMZ, Dong HF, Yang D, Raben N, Nagaraju K, Rosen A, Casciola-Rosen L, Härtlein M, Kron M, Yang D, Yiadom K, Dwivedi S, Plotz PH, and Oppenheim JJ. Histidyl-tRNA synthetase and asparaginyl-tRNA synthetase, autoantigens in myositis, activate chemokine receptors on T lymphocytes and immature dendritic cells. *J Exp Med* 196: 781-91, 2002.

How the body distinguishes between self-antigens and foreign antigens has been the central question in immunology for decades. We have investigated the recognition of self-antigens in autoimmune disease by studying the ability of autoantigens to interact with specific chemoattractant receptors and to induce the migration of different classes of leukocytes. Our observations suggest that autoantigens can recruit both inflammatory cells and specialized antigen-presenting cells, thereby connecting innate inflammatory and adaptive immune responses.

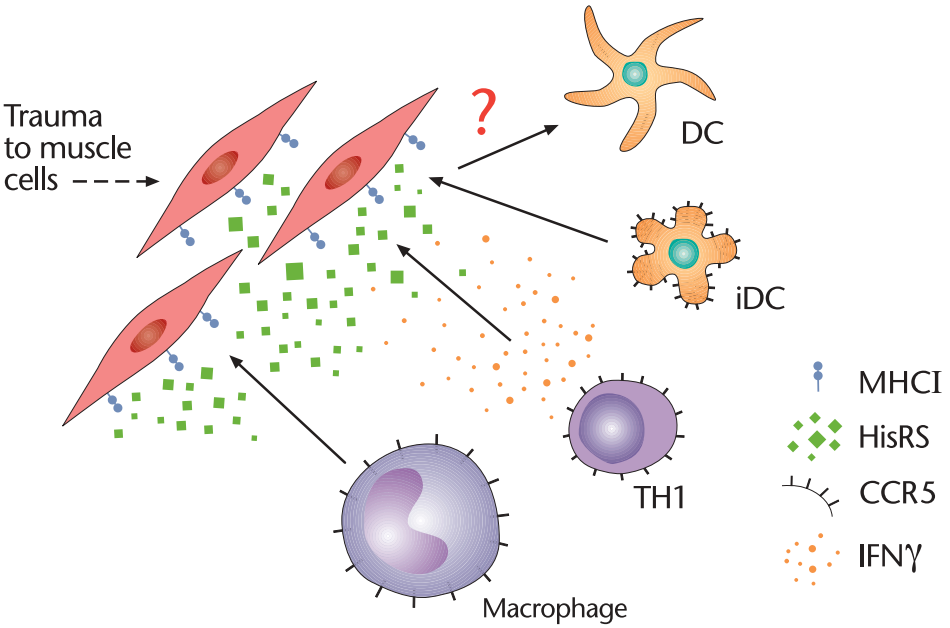

Figure 1. Proposed role of histidyl-tRNA synthetase (HisRS) in myositis. Recombinant HisRS (rHisRS) is a more potent chemoattractant (0.17 nM) than stromal cell-derived factor 1α (12.5 nM). Migration induced by HisRS is sensitive to pertussis toxin. CCR5-expressing cells, including immature dendritic cells (iDC), migrate to rHisRS and the amino-terminal peptide 1–48 HisRS. rHisRS desensitizes CCR5-expressing cells to CCL4 (MIP1-β) or CCL5 (RANTES)-induced migration. However, rHisRS *does not* compete with radiolabeled MIP1-β or *RANTES in a direct competition binding assay. The third and fourth extracellular domains of CCR5 are needed for rHisRS-induced chemotaxis. DC, dendritic cell; TH1, helper T cell 1; MHCI, major histocompatibility complex I; IFNγ, interferon gamma.

Patients with idiopathic inflammatory myositis experience debilitating muscle weakness. The most common autoantibodies in myositis are directed against one of the aminoacyl-tRNA synthetases, which are intracellular components involved in protein translation. The most frequent autoantibody, expressed by 15 to 25 percent of patients, targets histidyl-tRNA synthetase (HisRS). A smaller percentage of patients express autoantibodies directed to alanyl-, asparaginyl-, glycyl-, isoleucyl-, or threonyl-tRNA synthetases. Autoantibodies to tryptophanyl- and seryl-tRNA synthetases have been reported in a few patients with systemic lupus erythematosus or rheumatoid arthritis, but not myositis. The remaining aminoacyl-tRNA synthetases have not been shown to be the targets of autoantibodies.

The reasons for the selective generation of an autoantibody repertoire directed against a restricted set of autoantigens—in any autoimmune disease—have been the objects of much speculation. Molecular mimicry of a self component by a microorganism seems increasingly unlikely to be the explanation for myositis, and theories of generalized upregulation of immunity or downregulation of tolerance cannot explain the limited repertoire of autoantibodies found in autoimmune diseases.

It has been reported that human tyrosyl-tRNA synthetase, not a known autoantigen, is a chemoattractant for neutrophils and activates monocytes when proteolytically cleaved. This observation suggests that aminoacyl-tRNA synthetases, targeted by autoantibodies in myositis, might have chemoattractant and proinflammatory properties. Our studies showed that HisRS, the amino-terminal peptide 1–48 HisRS, and asparaginyl-tRNA synthetase (AsnRS) recruit mononuclear leukocytes by acting on specific chemokine receptors. Specifically, HisRS is chemotactic for CCR5-bearing cells and interacts with at least the third and fourth extracellular domains of the receptor, whereas AsnRS induces CCR3-expressing cells to migrate. Both HisRS and AsnRS chemoattract immature dendritic cells (iDC). In contrast, seryl-tRNA synthetase (SerRS) chemoattracts cells transfected with CCR3 but not iDC. HisRS and AsnRS are autoantigens in myositis, whereas SerRS has been identified as an autoantigen in a few patients with systemic lupus

erythematosus or rheumatoid arthritis, but never in patients with myositis. Several aminoacyl-tRNA synthetases that are not autoantigenic are not chemoattractants.

The potential of HisRS and AsnRS to recruit iDC to sites of muscle inflammation and the demonstrated presence of activated monocytic and lymphocytic cells bearing CCR3, CCR5, and co-stimulatory molecules in myositic muscle suggest that these tRNA synthetases themselves may participate in the initiation of an adaptive immune response that leads to the production of autoantibodies. It has been amply documented that antigens capable of binding to receptors on antigen-presenting cells are processed tens of thousands of times more efficiently than antigens that don't bind to receptors on antigen-presenting cells and are therefore more immunogenic than the non-binding antigens. We have observed that HisRS causes CCR5 to quickly become sequestered, presumably leading to processing. Thus, on the basis of their chemotactic activity for iDC, we suggest that HisRS and AsnRS initiate a cascade of immune events, beginning with antigen presentation to T cells and leading to the production of B cell-stimulating cytokines that results in the production of autoantibodies in patients with advanced disease.

Finally, although our observations do not fully delineate the immunological events leading to the production of autoantibodies, they do indicate the participation of iDC, potentially connecting the chemotactic activity to initiation of the adaptive immune response represented by autoantibodies (Figure 1). Our recent unpublished observations show that many autoantigens, involved in a variety of autoimmune diseases, are also chemoattractants for iDC. These studies suggest that self-antigens that can interact with receptors on iDC may play a direct role in initiating and promulgating autoimmune diseases.

■ **O. M. Zack Howard, PhD**
Staff Scientist
Laboratory of Molecular Immunoregulation
NCI-Frederick, Bldg. 560/Rm. 31-19
Tel: 301-846-1347
Fax: 301-846-7042
howardz@ncifcrf.gov

■ BIOTECHNOLOGY RESOURCES

Cell-free Protein Synthesis—An Old Dog with New Tricks

A key to clear understanding of the role of genes in an organism is to understand the functions of all its proteins (biochemical activities and protein–protein interactions) at the molecular level. Acquiring this knowledge will depend in part on the rapid expression and purification of proteins on a large scale and with high throughput. Accomplishing such rapidity will be far more difficult than the sequencing of genomes because the behavior of proteins is notoriously variable. Many molecular tools enable *in vivo* protein expression in host organisms such as *Escherichia coli* and eukaryotic cells. However, the labor required to express many genes is considerable and the ways to fail are numerous, including insolubility, toxicity to the host, and instability (e.g., proteolysis).

These considerations have led to attempts to bypass the intact host organism, and to use instead cell extracts that contain the essential transcription or translation factors and simply add the DNA template to the extract and let expression proceed *in vitro*. This strategy, called cell-free protein expression (CFPE), has several conceptual advantages. 1) Producing protein *in vitro* is more amenable to high-throughput methods because cells have already been grown and harvested (to make extracts). Since production can be done at a large scale, *n in vitro* reactions replace *n* cell cultures, *n* harvests of these cultures, and the preparation of *n* extracts. 2) Cell walls are no longer there to contend with, so adding enzymes, lipids, chaperones, membranes, protease inhibitors, or modified amino acids provides new opportunities for improving the yield or modifying the properties of the protein of interest. 3) The issue of toxicity of the expressed protein disappears. 4) Because cellular maintenance is not required in the cell-free system, almost all of the cell's energy possibly could be channeled to produce protein. It might appear that the cost of increasing the scale of expression would be higher with CFPE, but cells must be cultured and harvested and processed, whether the gene encoding the desired protein is present or not. Thus, at equal productivities (an important proviso), the costs of CFPE and *in vivo* protein expression should be independent of scale.

The NCI/SAIC Protein Expression Laboratory in Frederick has developed the most efficient CFPE system available today.

The idea of CFPE is not new. Typically, cells of a host organism (most commonly *E. coli*, wheat germ, or reticulocytes) are lysed and the components of the transcription and translation machinery are harvested by centrifugation. Recent improvements in the technology of CFPE based on *E. coli* extracts include preparation of the cell lysate in more concentrated form, removal of endogenous RNAs and amino acids during processing of the extract, addition of a novel energy-regenerating source and enzymes that

generate the ATP necessary to power the processes of aminoacylation and protein synthesis, and addition of exogenous phage T7 RNA polymerase and plasmid or PCR product DNA template containing the gene of interest transcribed from a T7 promoter. The combination of these improvements has dramatically improved the productivity of CFPE.

The NCI/SAIC Protein Expression Laboratory (PEL) in Frederick has developed the most efficient CFPE system available today. In a side-by-side comparison performed in the laboratory of Stuart Le Grice, PhD, the PEL technology produced about five times more HIV p66 protein than the commercial product did and the yield of HIV-1 reverse transcriptase p66 was almost equivalent in both *in vivo* and cell-free expression (Figure 1). Further, highly expressed genes such as green fluorescent protein (GFP) and chloramphenicol acetyl transferase (CAT) could yield up to 1 mg of protein in a 1-ml reaction; the yield of protein was essentially identical when fluorotyrosine was substituted for tyrosine, illustrating the opportunities for introducing modified amino acids into proteins. An optimized component system with a new ATP-generating source, 3-phosphoglycerate, increased protein yields almost twofold compared with the existing technology using phosphoenol pyruvate as the energy source (Figure 2).

We are using CFPE in collaboration with a number of NCI investigators. Stephen Hughes, PhD, is interested in producing soluble reverse transcriptase in the cell-free system by adding combinations of chaperones. Andrew Byrd, PhD, wishes to incorporate stable isotopes and modified amino acids into proteins for nuclear magnetic resonance spectroscopy. Dr. Le Grice seeks to place amino acid analogs at particular positions within HIV reverse transcriptase by using templates containing amber stop codons at those positions and adding suppressing tRNAs charged with the desired analogs. Dimiter Dimitrov, PhD, is interested in several proteins involved in cancer and AIDS. CFPE may be especially well suited to study protein folding, *in vitro* evolution, viral assembly, mRNA display, ribosome display, and functional characterization proteins in general.

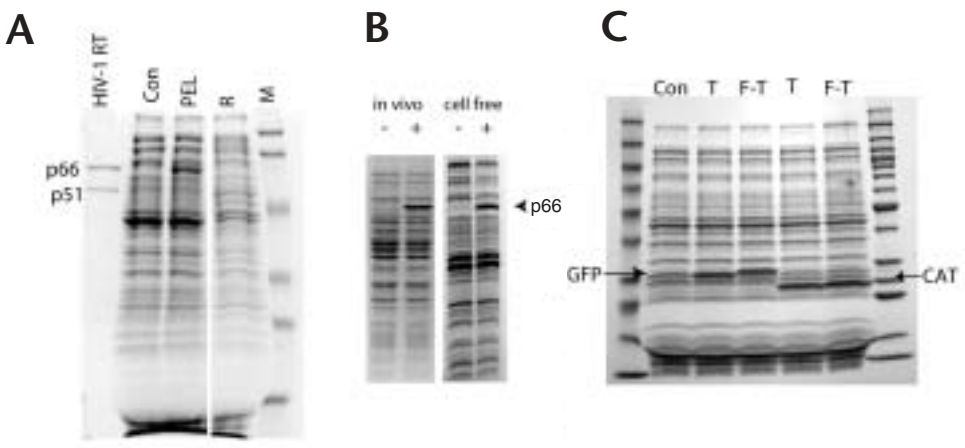

Figure 1. *A:* Higher levels of expression of the HIV-1 reverse transcriptase (RT) p66 subunit in the NCI/SAIC Protein Expression Laboratory (PEL) cell-free system relative to a commercial source (R). The lane without any plasmid DNA served as a control (Con). M, molecular weight. *B:* Level of expression of p66 under the control of a T7 promoter in *E. coli* cells (*in vivo*) and in the cell-free system (cell free). For *in vivo* cells, + and – indicate with or without induction; for the cell-free system, + and – indicate with or without template plasmid. *C:* Expression of green fluorescent protein (GFP) and cloramphenicol acetyl transferase (CAT) in the presence of normal tyrosine (T) or fluorotyrosine (F-T). The lane containing 19 amino acids without tyrosine served as a control (Con).

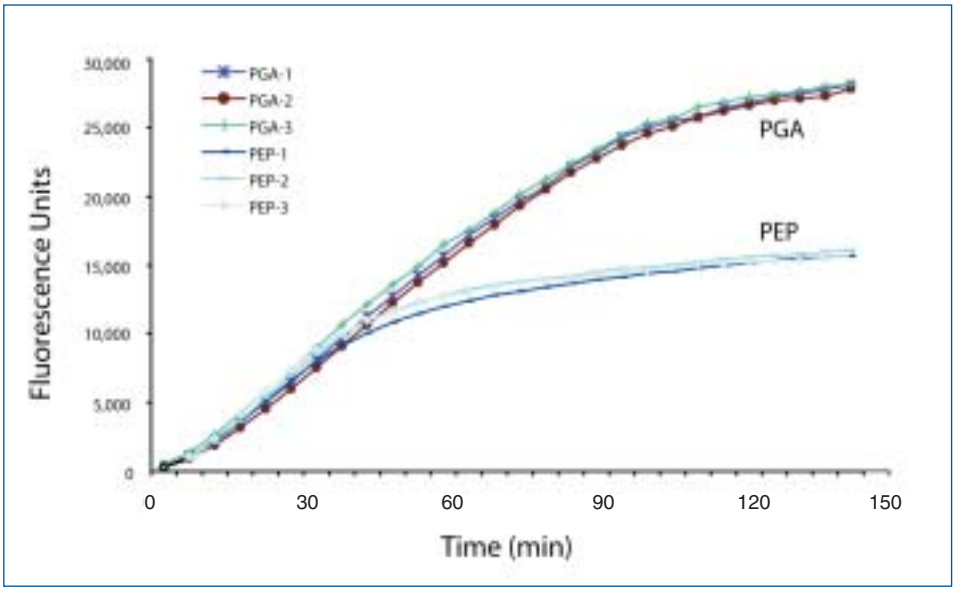

Figure 2. Real-time monitoring of expression of green fluorescent protein (GFP) in the presence of 3-phosphoglycerate (PGA) and the conventional phosphoenol pyruvate (PEP) as the energy source. The reaction is almost over by 45 min for PEP but continues for almost 2 hr for PGA.

To access this unique technology at a cost substantially less than commercially available systems, contact Dr. Chatterjee. For other services available from the NCI/SAIC Protein Expression Laboratory, see http://web.ncifcrf.gov/rtp/pel/.

■ **Deb K. Chatterjee, PhD**
Associate Director and Senior Principal Scientist
NCI/SAIC Protein Expression Laboratory
NCI-Frederick, Bldg. 327/Rm. 5
Tel: 301-846-6893
Fax: 301-846-6631
chatterjee@ncifcrf.gov

Reaching Out to Special Populations: CSSC Boosts Referrals to NCI Clinical Studies

The NCI Clinical Studies Support Center (CSSC) has been reaching out to minority populations and women in the Washington, D.C., area since its inception in 1998. Between April 2000 and December 2002 the Center conducted 81 meetings and events, of which 27 specifically targeted African Americans and 15 targeted Hispanics. (Other events addressed all community members.) Because of the success of CSSC's early outreach efforts, the Special Populations Outreach Pilot Program was initiated in January 2001 to inform residents about cancer clinical research.

The pilot program goes beyond media-based outreach strategies to employ innovative partnerships, educational forums, and culturally appropriate materials. It seeks to remove barriers and increase cancer trial referrals in areas of Washington, D.C., that have high concentrations of African American, Hispanic, and medically underserved residents (Wards 1 and 8). The program informs communities and the physicians who serve them about clinical research in general, and about specific cancer trials. Early results suggest this community-based approach is effective in fostering participation in clinical trials: calls to CSSC from the District have increased by 52 percent over the prior 2 years (Figure 1).

The Special Population Outreach Pilot Program's partnerships (Figure 2) allow NCI principal investigators to connect directly with minority communities and the physicians who serve them. By developing relationships with community clinics, health maintenance organizations, and other organizations that serve these communities, the program keeps abreast of opportunities to promote NCI clinical trials. As a result, clinical research information is delivered into the hands of people who talk to community members every day.

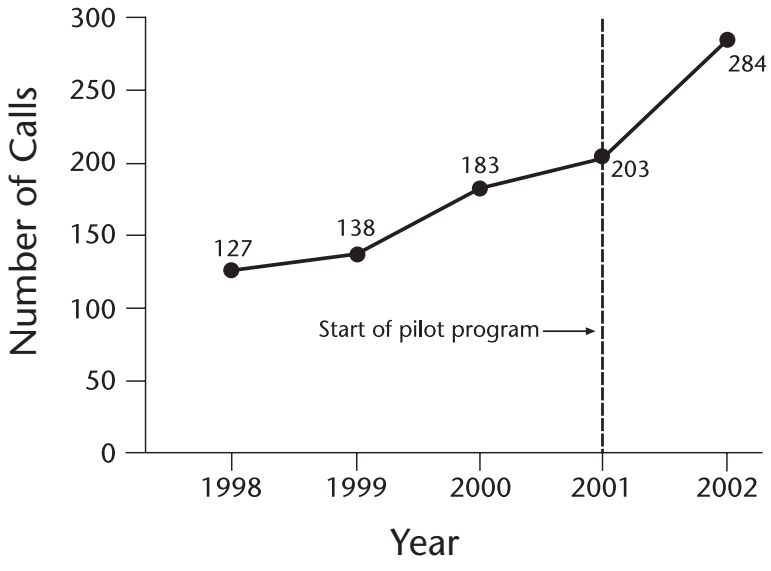

Figure 1. Calls from Washington, D.C., area residents to the Clinical Studies Support Center, 1998–2002.

- Alpha Kappa Alpha
- Association of Community Cancer Centers
- Breast Cancer Resource Committee
- Capital Community Health Plan
- Casa de Maryland
- Chartered Health Plan
- Congressional Black Caucus Convention
- Congressional Hispanic Caucus Convention
- DC Department of Health, Bureau of Chronic Disease
- Delta Sigma Theta
- Enfola Senior Center
- Greater Southeast Community Hospital
- Health Right, Inc.
- Howard University
- Howard University Women's Conference
- Intercultural Cancer Council
- La Clinica del Pueblo
- National Association of Hispanic Nurses
- National Black Nurses Association, Inc.
- National Hispanic Medical Association
- National Medical Association
- Omega Psi Phi International
- Public Health Association of the Greater Washington Area
- Spanish Catholic Center
- Unity Health Care, Inc.

Figure 2. Examples of Clinical Studies Support Center partner organizations.

As a launching activity, the pilot program mailed CSSC information to 651 physicians who were affiliated with three of these partners. Subsequently, the program placed articles in organizational newsletters, sent representatives to attend community events, and educated local physicians and community leaders about upcoming clinical trials. Educational presentations, often provided by principal investigators, have covered topics such as the referral process, study goals and criteria, and clinical trials in general. The program has also attended local events at churches, advocacy organizations, sororities, and fraternities. There, it has presented exhibits, distributed information about CSSC or specific studies, and discussed clinical studies with participants.

The program recently developed a brochure about clinical trials research, which is tailored for African American audiences. The brochure was based on focus group testing with African American residents of Washington, D.C., and Baltimore. It will be finalized and released in coming months. The success of the program suggests that community-based approaches to clinical trials recruitment may yield strong results if expanded to other sites.

CSSC, along with its Special Populations Outreach Pilot Program, is available to help NCI principal investigators promote their studies and accrue patients. CSSC can help with promotional activities, including brochures, mailings, events, and community outreach. To learn more, contact Bridget Robinson, CSSC Special Populations Coordinator, at 301-984-7191, ext. 1608 or Bridget.Robinson@matthewsgroup.com.

FROM THE DIRECTOR

The Medical Oncology Research Unit: A Cornerstone of Excellence in Clinical Research and Patient Care

This is the first of a two-part series focusing on the Medical Oncology Clinical Research Unit (MOCRU).

The Medical Oncology Clinical Research Unit (MOCRU) was established in 2001 under the leadership of Ronald E. Gress, MD, to provide investigators throughout the NCI with centralized access to clinical research expertise and resources in the CCR. Its mission is to act as a clinical partner to all intramural investigators, facilitating the delivery of laboratory discoveries into the clinical setting and establishing standards of excellence for clinical research and patient care. The MOCRU fosters collaborations between basic researchers and clinical investigators to expedite drug development, clinical trials, and data management. It is actively involved in the development or validation of novel biomarkers and clinical end points, as well as the application of intramurally developed technologies in the clinic. Additionally, the MOCRU offers unparalleled opportunities for physician-scientists training for careers in translational and clinical research.

[The MOCRU's] mission is to act as a clinical partner to all intramural investigators, facilitating the delivery of laboratory discoveries into the clinical setting and establishing standards of excellence for clinical research and patient care…. Additionally, the MOCRU offers unparalleled opportunities for physician-scientists training for careers in translational and clinical research.

Since its inception, the MOCRU has served as a focal point for promoting the standardization of clinical research in medical oncology in the CCR and integrating all medical oncology clinical research efforts originating in different laboratories, branches, and other NCI and NIH entities. The recent reconfiguration of the Unit's bioinformatics infrastructure illustrates this integrative function. In collaboration with Kenneth H. Buetow, PhD (Director of the NCI Center for Bioinformatics), Jo Anne Zujewski, MD (Medical Director, Clinical Research Operations) and others have developed the Cancer-Centralized Clinical Database (C3D), a patient-centered tool for data capture and management that permits data generated from clinical trials to be standardized and cross-referenced. The system has built-in quality assurance measures and will provide a platform through which clinical data can be integrated with laboratory data—a difficult goal, given the relative lack of standardization characteristic of clinical data. The database constitutes a critical component in the translational infrastructure linking laboratory and clinic.

The MOCRU infrastructure is designed to promote interdisciplinary collaborations among investigators, encourage the bi-directional flow of information between the bench and the bedside, and accelerate translation while providing optimal patient care and protocol

support. The partnership between Louis M. Staudt, PhD (CCR Metabolism Branch) and Wyndham Wilson, MD (head of the MOCRU Lymphoma Clinical Research Section) offers an example of a successful, long-term collaboration supported by the MOCRU. During the late 1990s, investigators in the Staudt laboratory identified two molecularly distinct subtypes of diffuse large B cell lymphoma using the Lymphochip, a microarray developed in the CCR with genes selectively expressed by B cells. Collaboration with Dr. Wilson led to the rapid exploitation of this discovery for patient care. Working with Seth Steinberg, PhD (head of the Biostatistics and Management Section) and other MOCRU-affiliated clinical research professionals, the Staudt/Wilson team has recently initiated a protocol to validate the principle that molecular profiling can provide clinically useful information. Using gene expression profiling, the trial will attempt to determine whether individual responses to standard treatment correlate with molecularly defined disease subtypes. A robust correlation between clinical course and gene expression patterns would provide evidence strongly supporting molecularly based approaches to diagnosis, prognostic assessment, and treatment selection.

The MOCRU consists of eight offices, two scientific cores, and eight clinical research sections (CRSs). Several primarily support career development for post-doctoral fellows and clinical research professionals. The educational program includes formal fellowship training in medical oncology under the direction of Barry Gause, MD, instructional programs in research nursing under the guidance of Anne Thomas, PhD, and recruitment/training programs for physician assistants and nurse practitioners under the leadership of Juan Gea-Banacloche, MD. Janet Jamison in the Protocol Support Office has initiated a new effort to provide regulatory guidelines and Good Clinical Practice education for physicians involved in clinical research.

The remaining MOCRU offices support various components of the clinical research program as a whole. Headed by Giovanna Tosato, MD, the Office of Scientific Review offers peer review of clinical trials and assesses ongoing studies in aggregate to provide a programmatic perspective of the CCR's clinical oncology research efforts. The Office of Translational Research, led by Jon Wigginton, MD, assists investigators interested in designing protocols, participating in ongoing trials, or establishing relationships with clinical collaborators. Complementing this function, the Protocol Support Office supports investigators and research teams throughout the lifetime of a protocol. It supports clinical trial design, assists research teams in satisfying Institutional Review Board requirements and federal regulations, and performs regular internal audits to ensure data integrity and compliance with Good Clinical Practice and regulatory guidelines.

The MOCRU infrastructure is designed to promote interdisciplinary collaborations among investigators, encourage the bi-directional flow of information between the bench and the bedside, and accelerate translation while providing optimal patient care and protocol support.

MOCRU CRSs constitute an "open door" that gives investigators initial access to clinical expertise, services, and resources. The sections provide expertise in disease areas and therapeutic approaches of interest to the CCR scientific community, including breast cancer, genitourinary and gynecological cancers, lymphoma, AIDS malignancies, vaccines, transplantation, immunotherapy, and clinical genetics. Two additional CRSs are being organized: one devoted to Phase I clinical trials and the second to lung and gastrointestinal tract malignancies. The CRSs provide loci for collaborations between individual investigators as well as among faculties and branches throughout the NCI. For example, Dr. Zujewski, head of the Breast Cancer CRS, is leading a breast cancer prevention initiative that involves partnerships with Barbara Vonderhaar, PhD, who heads a project to characterize normal breast biology, the Cancer Prevention Branch, the Genetics Branch, the Laboratory of Biosystems and Cancer, and the Division of Cancer Epidemiology and Genetics.

To facilitate translational research and maintain high clinical research standards, MOCRU scientific core facilities provide other avenues for collaboration during protocol development and implementation. The clinical pharmacology and pre-clinical cores, headed by Douglas Figg, PharmD, and Janet B. Trepel, respectively, sustain the critical translational moment when investigators move from characterizing drug effects in animal models to studying those effects in humans. Their staffs have expertise in assessing pharmacokinetics and pharmacodynamics and in developing novel assays required by specific protocol designs, among other facets of drug development. A molecular targets core is being planned to provide the specialized expertise and resources needed to conduct state-of-the-art clinical studies.

By training research professionals, establishing and maintaining standards for clinical research and patient care, and assisting intramural investigators throughout every phase of clinical trial conceptualization and execution, the MOCRU acts as the cornerstone of the CCR's translational research process. Its ultimate *raison d'être* is to deliver improved therapeutics to cancer patients—to prolong individual life, enhance individual patients' quality of life, and improve the public health.

To learn more about the MOCRU's mission, infrastructure, resources, and personnel, visit http://home.ccr.cancer.gov/mocru/.

■ **J. Carl Barrett, PhD**

ON THE TENURE TRACK

Brad St. Croix, PhD

Born and raised in Kenora, Ontario, Canada, Brad St. Croix, PhD, moved to the west coast to attend the University of British Columbia in Vancouver and graduated with a BSc in marine biology. During his studies at the University of British Columbia he developed a strong interest in genetics, cancer, and tumor angiogenesis. He went on to the University of Toronto where he completed his PhD in the laboratory of Dr. Robert S. Kerbel. His post-doctoral training was done at Johns Hopkins University in the joint laboratory of Dr. Bert Vogelstein and Dr. Ken Kinzler. While at Johns Hopkins, Dr. St. Croix began the first systematic search for genes that are upregulated in newly formed endothelial cells that line tumor vessels. Because most solid tumors depend on new vessel growth, or angiogenesis, for expansive tumor growth, the products of such genes are thought to hold enormous potential as targets for anti-angiogenic therapy of cancer. Using serial analysis of gene expression (SAGE), a technology developed in the Vogelstein–Kinzler laboratory, Dr. St. Croix identified 46 human tumor endothelial markers (TEMs). Recent efforts have focused on four novel cell surface TEMs because of their potential accessibility to blood-borne therapeutics.

Dr. St. Croix joined the CCR's Mouse Cancer Genetics Program in June 2002 as a tenure-track principal investigator. His work continues to focus on tumor angiogenesis with an emphasis on using mouse models for translational studies of TEMs. Using gene knockout technology, he hopes to determine the role of various TEMs, especially those on the cell surface, in tumor angiogenesis. His laboratory is also interested in identifying the interacting partners of TEMs, particularly those involved in novel signaling pathways. Dr. St. Croix continues to use SAGE technology to gain a more comprehensive knowledge of the genes involved in angiogenesis. The goal of his research is to use new molecular information on tumor angiogenesis to develop clinically useful agents for improved diagnostics and therapeutics of cancer and other angiogenesis-associated diseases.


Dr. St. Croix

Dr. St. Croix lives with his wife, 6-year-old son, and 5- and 2-year-old daughters. His favorite pets are fish, and he enjoys photography as a hobby. Most of all, he enjoys family life, especially building Lego with his kids.

ADMINISTRATIVE LINKS

New NIH Deputy Director Appointed
NIH Director Elias A. Zerhouni, MD, announced the appointment of Raynard S. Kington, MD, PhD, as the new Deputy Director of NIH. Dr. Kington has served as NIH Associate Director for Behavioral and Social Sciences Research and as Director of the NIH Office of Behavioral and Social Sciences Research since November 2000. He also served as the Acting Director for the National Institute on Alcohol Abuse and Alcoholism (NIAAA) from January 2002 until September 2002. For more details, go to http://camp.nci.nih.gov/admin/news/admin/200303/kington.htm.

Conflicts of Interest: Recusals
A recusal or disqualification is a method used to resolve an apparent or actual conflict of interest. A disqualified employee must sign a written statement reflecting the scope of the disqualification and the precise nature of the conflicting interest or activity. An *apparent* conflict of interest arises where an employee is involved in a particular matter involving specific outside parties (including individuals and corporate entities) and the circumstances are such that a reasonable person with knowledge of the relevant facts would question the employee's impartiality in the matter. An *actual* conflict of interest exists when the employee has (or would have) official responsibilities with an outside organization with which that employee has a financial interest (his or her own, or an interest which is imputed to the employee). For more information on circumstances in which a recusal or disqualification is required, go to http://camp.nci.nih.gov/admin/news/admin/200303/recusals.htm.

The A-76 Competitive Sourcing Program
The purpose of the Competitive Sourcing Program is to implement OMB (Office of Management and Budget) Circular A-76 to create cost savings for NIH. The A-76 Circular establishes procedures (cost comparison) for determining whether commercial activities should be performed under contract with commercial sources, or in house using government facilities and personnel. For information pertaining to the A-76 process at NIH, go to http://a-76.nih.gov/.

***CCR Frontiers in Science:* Online and Hard Copies**
Current and archived issues (PDFs) of *CCR Frontiers in Science* can be viewed or printed by going to http://ccr.cancer.gov/news/newsletter.asp. Please contact Ave Cline at acline@mail.ncifcrf.gov if you would like to receive additional hard copies for your group.