National Cancer Institute

HIV and Cancer Virology Faculty Meeting

February 4, 2002

Holiday Inn, Gaithersburg, Maryland

Draft Summary

"The mission of the HIV and Cancer Virology Faculty is to promote and facilitate basic, clinical, and epidemiological interdisciplinary research on HIV, cancer-related, and other viruses."

Welcome and Introductions

Faculty Chair James Goedert welcomed participants and noted that the meeting was divided into two major parts. The first part, open to only NCI HIV and Cancer Virology Faculty (principal investigators and staff scientists), involved faculty issues, including communications, meetings, mentoring, and recruitment. The opening session of the meeting also included a discussion of faculty goals and priorities. The second part of the agenda, which was open to all interested investigators, involved presentations on ongoing research in HIV- and cancer-related virology conducted by NCI researchers. The purpose of this second meeting of the HIV and Cancer Virology Faculty was to work in aggregate to identify and address key issues and concerns in HIV and cancer research at the NIH.

Faculty Issues

Communications

Communication issues discussed during the meeting focused primarily on identifying ways to improve and facilitate communication among faculty in a timely manner. E-mail remains the most efficient means of communication for notification of meetings and for reminders; however, Jim Goedert and others acknowledged, this is still a work in progress. The faculty e-mail address list needs to be completed and updated to ensure that all faculty members receive notices and reminders. Jim Goedert noted that plans to develop a separate centralized list of CCR and DCEG postdocs are underway.

The HIV and Cancer Virology Faculty is linked through NCI's Center for Cancer Research (CCR) web site (http://ccr.ncifcrf.gov/default.asp); information on faculty events may be found at http://ccr.ncifcrf.gov/events/ and http://www.palladianpartners.com/irpfaculties/default.htm.

<u>Follow-up/Action Items</u>:

- Investigators from the Bethesda, Executive Boulevard, and Frederick campuses should forward their e-mail addresses and the e-mail addresses of postdocs, fellows, and others (as desired) in their labs to Jim Goedert, who will work with Joan Hanley-Hyde in compiling master address lists. Investigators are encouraged to share lists with and forward notices to postdocs.

- ♦ Denise Whitby will provide Joan Hanley-Hyde and Jim Goedert with a Frederick-wide address list.
- ♦ Palladian Partners (contractor) will assist NCI in maintaining and updating the centralized master address list(s).
- ♦ Jim Goedert will e-mail the web address of the Virology Faculty web page to faculty members for whom he has an e-mail address.
- ♦ Faculty members interested in volunteering to assist in coordinating faculty-related web site topics and communications issues should contact Jim Goedert.

Meetings

Investigators are very interested in discussing research projects being conducted by investigators in NCI's various branches and laboratories. Formal meetings represent one means of facilitating these discussions. Ultimately, the purpose of the meetings is to generate new ideas and establish new collaborations, with the faculty as a venue for further discussion. Key issues raised by the faculty included deciding on the frequency with which the faculty should meet and the format of the group's meetings, and whether to open meetings to non-faculty investigators, postdocs, and others.

The faculty should meet at least once a year to chart its course for the future year and to discuss policy and related issues. Most attendees agreed that the faculty should meet an additional two to three times each year to discuss scientific issues. The Faculty Steering Committee suggested a variety of meeting formats at the PI retreat in January. Faculty members attending the February 4 meeting explored these options further. One option is to follow the example of the Lymphoma Faculty and keep meetings open to all NCI (or NIH) investigators and faculty, allowing greater opportunities for new collaborations and cross-fertilization of ideas. Another option is to keep the meeting attendance limited to faculty and NCI investigators only. Tenure-track and senior-level investigators should be encouraged to participate and make presentations first, followed by postdocs and fellows.

The agenda could be structured as the current meeting was, with each investigator having approximately 30 minutes for a presentation and 15 minutes for a question-and-answer session. Alternatively, investigators could talk for a much shorter period of time (e.g., 15 minutes) followed by 10 to 15 minutes of questions, which would allow for a greater number of presentations and more discussion.

Attendees discussed inviting tenure-track investigators to the next meeting and structuring the agenda so that each of approximately 10 researchers has 20 minutes to present and 10 minutes for follow-up discussion. Others considered having 10 speakers overly ambitious, with too little time for in-depth presentations and for interaction among attendees. Additional suggestions included adding more break time and time for informal discussions; having three short talks (approximately 1.5 hours total) clustered in a morning session, breaking for a long lunch, and having a second presentation session of three talks (an additional 1.5 hours). Some faculty stated that attending full-day meetings can be difficult and suggested offering half-day meetings in the future.

The call for speakers for future meetings could proceed as with the current meeting. Jim Goedert noted that a call for investigators to volunteer to present was sent out approximately 2 months in advance of the meeting date and that this notice elicited a strong response. The next meeting was tentatively scheduled for the first week of June. The Holiday Inn in Gaithersburg was considered a convenient location for staff from all three campuses.

Regarding the PI retreat held in Virginia, Jim Goedert reported that investigators overall were satisfied with the retreat as conducted. Most respondents supported the "mixed" or non-thematic organization of the poster sessions as a means of promoting interaction among all NCI PIs and thus building a stronger NCI community. Faculty also found the larger talks at the retreat to be motivating. Suggestions for future retreats included adding multidisciplinary roundtable sessions; adding poster-presentations to the poster sessions; and including some broad thematic poster sessions (e.g., bench research with translational opportunities).

Follow-up/Action Items:

- Faculty members interested in helping to coordinate future meetings should contact Jim Goedert or Joan Hanley-Hyde.
- The next faculty meeting is tentatively scheduled for early June.
- Faculty members should forward dates that may conflict with an early June meeting to Jim Goedert.
- Future meetings will be announced using a series of clearly marked notices.
- All NCI PIs, including non-virology faculty, will be notified about the next faculty meeting using a global e-mail message.

Mentoring

Results of survey questions regarding mentoring suggested that most faculty members (16 of 19 respondents) are interested in serving as a career mentor to a virology postdoc from another lab or branch, and most (12 of 19 respondents) would be willing to mentor a postdoc from a non-virology lab or branch. Faculty members (16 of 19 respondents) also supported convening a half-day roundtable or other type of meeting with NCI non-virology faculty. With a high level of support for these activities, which will help facilitate information exchange among investigators, the HIV and Cancer Virology Faculty will continue to discuss incorporating mentoring and broader-based meetings into its plans.

Recruitment

The HIV and Cancer Virology Faculty Steering Committee considers recruitment and retention of faculty to be a priority. The committee has recommended advertising for available postdoc positions not only to attract new scientists but also to increase recognition of the HIV and Cancer Virology Faculty. It suggested running joint ads (i.e., across labs) in a professional journal, such as *Science*; sharing applications among labs; and inviting candidates to make one presentation to all interested labs. This would allow for a more organized, coordinated, efficient, and cost-effective approach to recruitment than searches by individual labs. Joan Hanley-Hyde noted that applications and CVs of candidates being considered for joint interviews may be entered into NCI's "Stargazer" database for private viewing by PIs. Another strategy involves direct recruitment through colleges and universities.

Follow-up/Action Items:

- Faculty members should visit the virology web page and critique it for its content, particularly in relation to the HIV and Cancer Virology Faculty.
- Faculty members should review NCI's Stargazer system, which allows users to identify potential candidates for fellowship positions using a variety of search strategies and terms.
- Inquire whether the faculty steering committee can develop a draft advertisement for review by the faculty.

Goals and Priorities

Attendees briefly discussed the history of faculties at the cancer institute. In response to a challenge by the former NCI Director, Dr. Richard Klausner, an intramural working group was convened to examine establishing NCI faculties. The working group strongly recommended that NCI investigators strive to develop projects and conduct research unique to the NCI that would stand out from other ICs, such as collaborative projects between basic research scientists and epidemiologists. Some of the NCI faculty goals and priorities might include identifying and developing long-term projects, conducting high-risk research, recruiting new faculty, and establishing collaborative partnerships with other faculties. Funds to support certain faculty efforts, such as meetings and retreats, are available through the Director's reserve.

HIV and Cancer Virology Faculty members were surveyed directly about goals and priorities. Jim Goedert grouped survey results and comments into three major categories: Internal communications, recruiting, and other. Comments indicated that the faculty has had a good start but that there is room for improvement, particularly with respect to becoming a more cohesive group with common goals of developing novel research ideas, establishing new collaborations, enhancing the value of HIV and cancer virology research at NCI, and improving recruitment and retention of quality investigators. Achieving these goals will require thoughtful planning, solid communication, and time.

One concern raised by the faculty was that the lower attendance at the current meeting, compared with previous meetings, may be a result of not being as well-defined or as cohesive a group as is needed. To attract and retain more investigators, the group needs to identify and promote the advantages of being a member of this faculty and to have greater continuity. It may be instructive to poll those who did not attend the February 4 meeting as to the reasons for their absence. Attendees noted that there was some confusion about the current meeting, including whether to bring postdocs and fellows. In addition, some faculty did not receive notices about the meeting, which underscores the importance of having and maintaining up-to-date master lists of faculty members and other investigators.

Attendees discussed the importance of interacting with non-NCI virology faculty in semi-formal settings, such as the current faculty meeting. A suggestion was made to invite PIs from NIAID to the next HIV and Cancer Virology meeting as a way to initiate this process. Other NIH groups that the HIV and Cancer Virology Faculty may pursue further include the Immunology Faculty, the Immunology Interest Group, the AIDS Interest Group, the Vaccine Research Center (VRC), and the Virology Interest Group. (The primary difference between a faculty and an interest group is that a faculty remains associated with a home institute or center, whereas an interest group often is broader based and draws investigators from a number of ICs.) The Virology Interest Group, which is led by Allison McBride at NIAID and currently has about 40 members, meets every Tuesday at 1 p.m. on the main NIH campus in Bethesda. The group has a web site, and its interests are relatively broad, extending beyond HIV. The Immunology Faculty also meets each Tuesdays and offers a weekly video link of investigators in Bethesda, Rockville, and Frederick. Of these groups and faculties, investigators associated with the VRC and the HIV and Cancer Virology Faculty may have the most in common. Attendees pointed out that the greater immunology community at the NIH has existed for about 25 years, long before either the faculty or interest group was established, leading to a strong, cohesive immunology following at the NIH.

Additional consideration was given to identifying how the faculty could move beyond being only a discussion group and move toward developing and supporting new and bold initiatives. Attendees emphasized the importance of being a more cohesive group before moving into new territory. The faculty may wish to identify both short- and long-term goals and to serve as a springboard for pursuing those goals in support of HIV and cancer virology research.

Follow-up/Action Items:

- ♦ Discuss and compare the goals and visions of the HIV and Cancer Virology Faculty with those of other faculties and interest groups. With this feedback in hand, identify and pursue ways to interact more closely with allied faculties and interest groups.

Scientific Presentations

Natural killer cells as a viral reservoir for HIV in patients (George Pavlakis, M.D., Ph.D.)

The role of natural killer (NK) cells as a reservoir for HIV in patients has not been studied extensively. However, research suggests that factors critical to the entry of HIV-1 into a variety of cells (e.g., CD4+ cells, lymphocytes, monocytes) *in vitro* also may influence the infectivity of certain subsets of NK cells. These factors include the CD4, CCR5, CXCR4 receptors. To understand the pathogenesis of HIV infection fully, Dr. Pavlakis commented, scientists must study the range of cells that are infected by or susceptible to infection by HIV.

Dr. Pavlakis reported that a non-T, non-monocyte leukocyte subset of cells with NK markers showed productive infection by HIV *in vivo* and *in vitro* in the presence of CD4 and HIV co-receptors. This cell population, CD3-CD56+CD16+CD4+, which represents approximately 1 percent of the total NK cell population in humans, has not been well studied but appears to be important to HIV infectivity. CD56+CD16+CD3- NK cells are cytolytic cells that participate in innate immunity. They lack surface receptors that recognize specific antigens but do help eliminate cells that are "missing self"; they also possess anti-tumor activity.

Experiments conducted in Dr. Pavlakis's lab by Antonio Valentin indicate that some of the peripheral blood mononuclear cells (PMBCs) infected *in vitro* with HIV have NK cell markers, as opposed to T cell markers. (The lab uses fully infectious GFP-producing HIV-1 clones in its studies.) The HIV clones used in these experiments appear to infect cells through either the CCR5 (R5) or the CXCR4 (X4) co-receptor. FACS analysis of productively infected cells revealed several lymphocyte subsets, including T cells (CD3+; the majority of the infected cells), true NK cells (CD56+), and T cells with NK markers (referred to as NK/T cells). Approximately 10 percent of the HIV-infected cells were either true NK cells or NK/T cells.

To obtain as pure a population of NK cells as possible for further study, Dr. Pavlakis, Valentin, and their colleagues fractionated PMBCs from healthy human subjects using a series of separations; CD14+ cells (monocytes) were extracted first, followed by CD3+ (T cells), and then CD56+ (NK) cells. The NK cells accounted for 5 to 8 percent of the PMBCs derived from healthy persons. The resulting CD56+ cell population was about 96 percent pure NK cells, with less than 0.4 percent of the cells identified as either T cells or monocytes. These cells were then infected productively with HIV-1 clones in tissue culture. Results showed that the infection of primary NK cells by HIV-1 is dependent on the CD4+ *and* CCR5 co-receptors. Infection of these cells was blocked in the presence of CD4 monoclonal antibody.

Further analysis of the NK cell compartment indicated that 2 percent of the CD3-CD56+ cells derived from PMBCs of healthy persons possessed both the CD4 and CCR5 co-receptors and thus should be infect able by HIV, as demonstrated. Between 1 and 4 percent of the NK pool had both the CCRX4 and the CD4 co-receptors. Thus, the fraction of HIV-infectable NK cells in healthy individuals is small (i.e.., 2 percent NKs of the 5 to 8 percent PMBCs). However, Dr. Pavlakis pointed out, the NK-infectable compartment is augmented in HIV-positive persons. CD4+ NK cells appear to account for a maximum of 6.5 percent of the total NK pool in healthy individuals, compared with 21 to 39 percent in some HIV-infected persons treated with HAART. In addition, the virus appears to remain inside the NK cells of infected persons, even in the presence of the most potent antiretroviral regimens available. These findings suggest that certain populations of NK cells may play a more important role in HIV infection and disease than previously thought.

Immunophenotypic analysis of CD4+ NK cells revealed the presence of CD16 marker, which is the Fc_{δ} receptor found in other NK cells. They are CCR5+ and CXCR4+ but are T cell receptor negative. These cells also are negative for the CD14 (i.e., lack monocyte markers), CD19, CD80, and CD85 receptors. They express KIR receptors in culture, which are further indication of their NK classification. Additional studies confirmed CD16 and KIR expression *in vitro* by CD4+ NK cells but not by T cells from the same individuals.

The next step involved experiments designed to demonstrate that HIV-positive persons have HIV-infected NK cells. An examination of cells from 24 infected patients revealed virus present in the CD3+ (T) cells and the CD3-CD56+ (NK) cells of all patients. Dr. Pavlakis reported that the number of copies of viral DNA in the NK compartment was substantial. The next step for Dr. Pavlakis's group involved investigating how the NK compartment behaves over time. To do this, the team studied samples from 10 HIV-infected patients, from pre-HAART, initiation of HAART, and through 2 years on HAART. In general, as expected, plasma viral load drops from detectable to undetectable following initiation of HAART. Viral DNA was detected in both the T cell and NK cell compartments before and after HAART, despite a decrease in plasma viral load. There was a gradual decline in viral DNA in T cells and NK cells over 2 years, but the virus never became undetectable in either compartment. Results indicated that that up to 50 percent of HIV DNA in some patients was attributable to the NK compartment. Additional statistical analysis of the data suggested that virus detected as DNA in the NK compartment persists as long as in T cells. Further studies and analyses are planned.

Differences in the number of cells that are potential HIV targets were found in healthy controls compared to infected patients. Dr. Pavlakis noted that in a study of a small group of controls (n = 5) and patients (n = 5), most of the CD4+/CCR5+ cells in controls were T cells. In contrast, in patients, the T cell targets decrease considerably, and the NK cell potential targets increase.

In brief, Dr. Pavlakis concluded that NK cells of HAART-treated patients are persistently infected *in vivo*; the distribution of innate immunity effector cell changes in HIV-positive persons is reflected in increases in CD4+ NK cells and in gamma-delta NK/T cells (unpublished data); and changes in cell compartments may be critical to disease progression and treatment. The new NK cell subpopulations that express the CD4, CCR5, and CXCR4 co-receptors warrant further investigation. Dr. Pavlakis noted that the key person on this research is Antonio Valentin. In response to a question about whether HIV-positive patients simply have an increased production of CD4+ cells, Dr. Pavlakis stated that it is not clear. One hypothesis that CD4+ cells are part of a developmental phase of NK cells is being tested by Dr. Pavlakis in collaboration with researchers at Frederick.

An attendee asked whether the greater absolute number of CD4+ cells in HIV-infected persons, compared with healthy individuals, suggests that the cells are induced to replicate via a bystander mechanism in HIV-infected patients. Dr. Pavlakis commented that if these cells are precursors to mature NK cells, a bystander mechanism is possible. However, the exact mechanism is not known. He noted that true NK cells develop extrathymically. With respect to studies and results in other species, Dr. Pavlakis stated that his group is examining NK cell responses in infected macaques and mice. He reported that they have found similar subsets of NK cells in both animals. Thus, although there is wide variability among the NK markers in different species, CD4+ appears to be stable in a small subset of NK cells; this may have functional significance.

In response to follow-up questions about the increase in infectable NK cells in HIV-positive patients versus healthy patients, Dr. Pavlakis stated that it appears that T cells and certain NK cells (CD56+CD3-) are potentially infectable by HIV. In healthy, uninfected persons, approximately 3.5 percent of NK cells are potentially infectable; in contrast, up to 50 percent of NK cells in HIV-positive patients are potentially infectable by the virus. Many HIV-infected patients have a significantly depleted T cell population, which, in turn, alters the proportion of NK cells within the infected group; in addition, the number of NK/T cells increases dramatically, although the proportion of all NK cells remains relatively constant. Thus, the decrease in T cells changes the NK compartment and with it, potentially the type of cells that are predominantly infected also appears to shift. Studies on the possible downregulation by CD4 have not been conducted, Dr. Pavlakis noted.

One attendee inquired about the lifespan of NK and NK/T cells, adding that if NK and other cells serve as a reservoir for latent virus, are these cells also long-lived? The exact lifespan of these cells is not known, but in the presence of a continuous infection, there could be a small population of long-lived cells and/or a component of cells infected *de novo*, even with HAART. Researchers are examining these possibilities using a two-pronged approached: by developing improved therapies that target all infected cells, and by comparing viral genotype following initial infection (or pre-HAART) with that of "rebound" virus after therapy is terminated. Additional genetic studies have been proposed. The study of innate immunity in relation to HIV/AIDS is a new but growing field with many gaps in knowledge. Dr. Pavlakis has not yet studied any other chronically infected controls but is working on obtaining cells from other populations to include in future investigations.

When asked about the role of IL-12 as a primary cytokine that might induce NK cells, Dr. Pavlakis stated that CD4+ NK cells exposed to IL-12 *in vitro* are not induced but they do show increased CD56 expression. Additional experiments may prove informative. Another attendee asked whether CD4+ NK cells produce perforin. Dr. Pavlakis replied that perforin has not been tested extensively; however, one experiment in NK cells has detected perforin. Conducting functional analyses would be preferable and are in progress, but such experiments are time consuming and complex.

Role of dendritic cells in the immunopathogenesis of HIV disease (Andrew Blauvelt, M.D.)

Dr. Blauvelt's lab currently is focused on modeling the events of sexual transmission of HIV and to understand the biologic events that occur during this transmission. Dr. Blauvelt and his colleagues have used a variety of tissues in these studies, including skin explants, vaginal mucosal biopsies, and foreskin. Regarding virus transmission, Langerhans cells (LC) within genital epithelial surfaces appear to be the initial targets for HIV. LLC are present in all epithelial layers, and they perform the same function in genital epithelia as they do in skin in that they are the primary immune sentinels at these surfaces. In this role, LC survey for bacteria, viruses, fungi, antigens, and other foreign agents. LC in vaginal mucosa are CD4+ and CCR5+.

This area of research is somewhat controversial, Dr. Blauvelt noted, in part because examination of vaginal tissue from macaques within 1 to 3 days after SIV exposure (in some studies) has not found a role for LC as a an initial target for SIV. However, the tissue may have been examined too long after exposure. LC respond to antigen and migrate out of tissue and into the draining lymph node within 4 to 6 hours after exposure; thus, some previous macaque studies appear to have missed this window of opportunity.

In one published study on an explant model, Dr. Blauvelt's group took skin from healthy volunteers using a suction blister technique to separate epidermis from dermis. In this case, the blister roof was a pure epithelial sheet, consisting entirely of keratinocytes and about 2 to 3 percent LC without any blood cell contamination. The skin was then draped over droplets of various strains and concentrations of HIV for 2 hours of exposure, after which the virus was washed away. Next, the skin was floated in a culture medium, causing the LC to migrate from the epithelial explants to the bottom of the wells over a 2- to 3-day period of time. The amount of HIV in LC was then quantified (using intracellular HIV p24 staining and flow cytometry). Representative results from one subject were shown and indicated that all of the LC were HLADR+ at day 3 after HIV exposure and that nearly 5 percent of the cells were infected with virus.

Similar experiments using explants from 62 healthy persons revealed that the number of LC that stained positive for p24 was highly variable. Subsequent testing showed that the variability was not due to the assay itself, but rather resulted from different CCR5 genotypes. Dr. Pete Zimmerman, a geneticist at Case Western, joined the NCI researchers in doing complete CCR5 genotyping (of the important polymorphisms known to influence HIV disease) in all of the healthy volunteers. Results of the genotyping suggested that the delta32 locus is important to HIV infectability and susceptibility; the percent LC infected was higher in wt-wt individuals than in individuals who were heterozygous for the delta32 mutation. This finding was statistically significant with a p value of 0.016 (n = 62 healthy controls). None of the 62 persons studied thus far were homozygous for this mutation, Dr. Blauvelt noted.

One goal of Dr. Blauvelt's research is to develop microbicides that act at HIV's initial entry or contact site. A primary goal or this work involves developing a topical microbicide that will prevent HIV transmission and will block HIV co-receptors. Agents currently under investigation are chemically modified (CM)-RANTES analogues and combination analogues that also have a CXCR4 inhibitor. Dr. Blauvelt's lab has been testing the efficacy of a panel of these novel microbicides using explant model systems before proceeding to animal and clinical studies. One analogue, TBA-RANTES, has shown a dose-dependent inhibition of R5-HIV infection of LC; inhibition is close to 100 percent at a concentration of 100 nM. Within-person comparative tests of 10-nM doses of a series of analogues demonstrate that PSC-RANTES and UCB-RANTES are the most potent inhibitors of HIV infection of LC.

Summarizing the status of research on LC and HIV, Dr. Blauvelt noted that R5-HIV infection in single LC can be detected and quantified following *in situ* exposure; infection levels are influenced by CCR5 genotype; infection can be blocked in a dose-dependent fashion; and LC infection cannot be blocked *in vitro* using C-type lectins. In conclusion, the data suggest that RANTES analogues can efficiently and persistently block R5-HIV infection in LC and other cell types and in other cultures; in addition, cumulative data indicate that PSC-RANTES blocks

CCR5 well and is effective for an extended period of time (i.e., up to 24 hours).

In response to a question about controlling for capture with respect to LC infectivity, Dr. Blauvelt noted that infection of the LC is completely blocked by blocking CCR5 and/or CD4; however, blocking HIV-C-type lection interactions (e.g., with mannan) fails to interfere with LC infection. Thus, it appears that HIV is not captured by LC, which is an unexpected finding.

Regarding *de novo* markers, Dr. Blauvelt stated that the current models do not provide a sufficient number of cells to study phenotype and infectivity in the same person. However, preliminary results suggest a strong correlation between CCR5 genotype and p24 positivity. A compound genotype that confers marked protection against HIV infection (wt-delta32, A/G polymorphism in a CCR5 promotor at –2459) is being analyzed further. Seven of the 62 healthy volunteers studied have this combination; tLC isolated from these individuals were very difficult to infect. Several studies indicate that other cell types with the same wt-delta32, A/G polymorphism showed reduced CCR5 expression.

Dr. Blauvelt's presentation continued with a discussion of experiments designed to investigate the impact of HIV infection and disease progression on antigen presenting function of dendritic cells (DC). Results of early studies suggested little effect of HIV on DC immune function; however, technical difficulties, such as the challenges associated with obtaining a sufficient number of cells, most likely limited these studies. One hypothesis suggests that HIV decreases IL-12 production, increases IL-10 production, decreases MHC class II+ cells, and allows monocytes/macrophages to become a more immune-tolerant APC rather than immune-stimulating APC. However, the literature on whether HIV infection impairs DC function in a similar manner is mixed at best, in part because labs often define DC differently, DC can be derived from different sources, and many of the infection protocols produce only small numbers of infected DC.

The NCI researchers revisited this issue to assess and clearly define the phenotype and the immunologic function of HIV-infected DC using new methodologies. The assay used monocyte-derived GM-CSF, IL-4-treated DC, which is now standard for working with and generating large numbers of DC *in vitro*. Adherent PBMC were grown for 1 week and then purified to 99 percent, exposed to a prototypic R5 or X4 isolates, and returned to culture. On day 10 after infection, cells were extracted and phenotyped using double-staining for p24-positive DC. Dr. Blauvelt noted that one of the most striking findings was that the p24-positive DC had marked downregulation of CD4 on the cell surface. Following this discovery, the researchers decided to enrich the HIV-infected DC population by removing the CD4+ cells remaining at 12 to 14 days post-infection. This enrichment yielded a purified population of DC in which 80-plus percent of the cells were HIV-infected and p24-positive. Dr. Blauvelt pointed out that cells are no longer viable after p24 staining; thus, alternative methods using a cell surface marker were needed to conduct functional analyses on viable cells.

Phenotypic analysis of the cell populations generated using these methods indicated that many important immunologic markers (e.g., MHC II, CD80, CD86) are not downregulated on HIV-infected DC. MHC class I is downregulated slightly, and CD4 and CD1a (an MHC class I-like molecule) are downregulated more significantly, by approximately 80 percent and 60 percent, respectively. Functional studies demonstrated that the enriched DC were impaired with respect to their ability to stimulate alloantigens (e.g., using an allo-MLR assay). In addition, DC enrichment led to a progressive decline in the infected cells' ability to stimulate T cells, when compared with uninfected and unenriched infected DC; the HIV-infected, enriched DC also produced less IL-2.

Dr. Blauvelt outlined several possible mechanisms of impaired APC function. Initially, researchers speculated that virus was being transmitted from the HIV-infected or –exposed DC to the T cell, causing impairment of the T cell's ability to proliferate and produce IL-2. As additional data accumulated, other hypotheses were considered. Phenotypic modification of DC was ruled out based on negligible changes in MHC class II, CD80, and CD86. The ability of infected DC to induce apoptosis, the direct infection of T cells, and alterations in cytokine production also have been postulated.

To study surface molecules of HIV-infected DC further, Dr. Blauvelt's team fixed DC prior to the MLR assay; T cell stimulation by DC remained normal under these conditions, which was consistent with the MHC class II, CD80, and CD86 findings. Thus, the surface of the HIV-infected DC appeared to be intact. With respect to cytokine production, the investigators found increased IL-12 production and decreased IL-10 production in the infected DC populations, in conjunction with poor stimulation of T cells. Additional experiments showed no difference in apoptosis between infected and uninfected DCs. In testing the hypothesis that virus was being transmitted from the HIV-infected or –exposed DCs to the T cell, the researchers found that antiretroviral drugs (i.e., a protease inhibitor, JE, plus ddI) blocked p24 production in DC-T cell co-cultures; however, the drugs failed to reverse this T cell stimulation defect. Further investigation revealed that T cell proliferation was restored in the presence of soluble CD4 (sCD4); the restoration of impaired T cell function was discordant with the amount of virus present. Dose-response studies showed a direct association between T cell proliferation and increasing concentrations of sCD4. The investigators also found that sCD4 did not affect viral load and that the antiretrovirals tested did not inhibit gp120 production in the co-cultures.

Dr. Blauvelt concluded his presentation by noting that highly enriched populations of HIV-infected DC could be readily and reproducibly generated and that alloantigen presenting capacity of infected DC was impaired, most likely as a result of increased production of gp120. Translating these findings to an *in vivo* scenario, the investigators have hypothesized whether the persistent cellular immune dysfunction in some HAART-treated persons is related to gp120 production by infected cells that are not affected by antiretroviral medications. The combination of HAART and sCD4 warrants further investigation as a means of overcoming this serious clinical problem and as a way to stimulate immune function as well.

Role for mucosal vaccines against HIV (Jay Berzofsky, M.D., Ph.D.)

Dr. Berzofsky noted that much of the vaccine research in his lab has focused on mucosal transmission of a recombinant vaccinia virus in mice, which is used as a surrogate for HIV infection in these animals. Studies also have involved challenging rhesus macaques with a pathogenic strain of SHIV.

The researchers' work on peptide vaccines that use defined epitopes as model vaccines began in the mid-1980s. In the mouse, regions defined as "cluster peptides," which contain clusters of overlapping helper epitopes that have with multiple MHC classes in mice, humans, and macaques, have been targeted for vaccine development. The source of these clusters is gp160. Further studies in several strains of mice showed that a portion of the V-3 loop, in addition to being a neutralizing agent, is an immunodominant CTL epitope also known as p18 (peptide 18). A variety of prototype vaccine constructs have been made using the helper epitopes and the CTL epitope.

Subsequent experiments compared the immunological responses to mucosal versus subcutaneous immunizations. Several mucosal routes were examined, and response was measured using Peyer's patches, splenic response, and CTLs. The greatest response was observed using intrarectal muscosal vaccination, which produced a cutaneous and a systemic response. SC immunization gave only systemic CTLs. Tests of the efficacy of mucosal immunization against intrarectal challenges of recombinant vaccinia (WR) virus in mice demonstrated significant protection by the vaccine. The protection was specific to gp160 and produced a three to four log drop in viral load immediately and was conferred in animals challenged up to 6 months after immunization.

Efforts to abrogate this protection by treating mice with anti-CD8 antibodies immediately before the viral challenge were successful, suggesting that protection is dependent on CD8 cells. It was unclear, however, whether the CD8 cells acted locally. Prior studies had shown that mucosal immunization produced both local and systemic (splenic) CTL responses, whereas the subcutaneous vaccine gave only a systemic response. Experiments in which animals were immunized subcutaneously or intrarectally demonstrated protection only in animals given the mucosal vaccine. These findings indicated that a mucosal (local) CTL response at the site of virus transmission was required for protection. This principle may have general applicability to protecting against HIV and other viral infections.

The next series of experiments demonstrated that treating mice with anti-IL-12 prevented the production of CTLs. In subsequent studies, intrarectally vaccinated and virus-challenged mice were given IL-12, which, in turn, enhanced CTL activity and protection against infection. The addition of IL-12 produced a six-log reduction in viral load in these animals, compared with a four-log reduction in animals receiving only the peptide vaccine, Dr. Berzofsky noted. A synergistic enhancement of CTL induction has been observed in systemically immunized mice with the combination of GM-CSF and IL-12. This synergistic effect required a round of at least four immunizations; two immunizations produced negligible protection. The findings overall suggest that the combination of four mucosal (intrarectal) immunizations plus GC-CSF plus IL-12 afforded the best protection and the strongest immune response against viral exposure.

Dr. Berzofsky's group also has tested the responses to and the efficacy of peptide vaccine constructs in Rhesus macaques. In this case, the mucosal adjuvant was a mutant, heat-labile, nontoxic strain of *E. coli*, rather than the cholera toxin used previously; the mutant *E. coli* adjuvant (mutant LT) vaccine, which was less IL-12 dependent than the vaccine used in mice, also induced a stronger CTL response. These findings were consistent with results from other laboratories. The vaccine construct used in macaques included several of the helper *env* epitopes recognized by mice and macaques; however, the V-3 loop epitope in the mouse vaccine was substituted with CTL epitopes *gag*181 and *pol*143 in macaques that were *mamu*-A*01-positive.

The first study included three groups of macaques: a control group that was immunized intrarectally with the mutant LT adjuvant alone (group A); a group that received all four peptides sc (group B); and a group that received all four peptides with mucosal adjuvant intrarectally (group C). All animals were given two cycles of four immunizations per cycle; biopsies were collected in each cycle for immunologic studies. All animals also received a vaccine boost 2 weeks prior to a challenge with a pathogenic strain of SHIV, a chimeric virus that expresses the *gag*181 and *pol*143 from SIV 239 as well as the HIV *env* genes from which the helper epitopes are derived. Results indicated that three animals had target-specific CTL lytic activity. Four of five responded to at least one epitope following intrarectal immunization. Response to the sc immunization (group B) was seen in the local axillary lymph nodes of all four animals in the group and in distant sites in two of the four macaques in this group. Animals in the adjuvant only group (group A) showed no response. Results indicated that the helper epitopes generated the strongest overall response (as measured by T cell proliferation associated with specific epitopes); however, animals with the greatest helper response also had the most significant CTL activity ($r = 4.87$, $p = 0.02$). Variability in MHC class II produced the variation in CTL response.

Next, 10 macaques were challenged intrarectally with a pathogenic strain of SHIV; two animals, including one in the control group, failed to become infected at all, and one animal became sick and had to be removed from the study. Animals were followed for 200 days after the virus challenge. Unlike the mice, none of the macaques initially was protected against infection based on plasma viral RNA levels and low CTL activity. However, at 60 days post-challenge, plasma viral load in intrarectally immunized animals dropped below the level of detection, in contrast with the other animals. The intrarectally immunized monkeys also had no opportunistic infections and had higher CD4 counts than animals in the other two groups. Statistically significant differences in nonparametric measures were found between the subcutaneous and intrarectally immunized groups; peak viral loads did not differ significantly between groups, however.

The reason for protection against viremia but not against transmission in following intrarectal (mucosal) immunization is not clear. Dr. Bezoksky noted that other investigators have shown that the major reservoir for SIV infection is in the gut mucosa, where CD4 levels are very high. There also appears to be a predilection for the virus to replicate in the gut. Thus, if mucosal immunization is superior to other routes in inducing mucosal CTL, as seen in the mouse, then mucosal immunization also might be more effective in eradicating a primary reservoir for virus replication. To test this hypothesis, the macaques were euthanized and necropsied on day 200. Analysis of tissue from the gut indicated that viral loads in the colon of controls and animals immunized subcutaneously were one to two logs higher than in animals given the mucosal (intrarectal) vaccine. The reduction of virus in the gut, in conjunction with mucosal CTL activity, may prevent the systemic viremia seen in the controls and sc-immunized animals.

A key conclusion to draw from these findings is that mucosal immunization and mucosal CTL induction are more effective than systemic immunization at controlling SIV/HIV infection in a primate. The efficacy of mucosal immunization appears to be due in large part to the reduction of viral load in the major reservoir for viral replication, rather than to prevention of virus transmission. These insights may be instructive in developing a mucosally administered HIV vaccine for humans.

Current research involves analyzing tissue samples for cytokine profiles in gut and CD8 antiviral factors. Further primate studies using novel vaccine epitope constructs and cytokine-enhanced adjuvants are planned.

Dr. Berzovsky noted that Dr. Igor Bellicov has been a key investigator in this research.

In response to a question, Dr. Berzovsky stated that animals in the studies described above were vaccinated and challenged via the intrarectal route. Studies in which mice are vaginally immunized and challenged are underway. Similar studies in monkeys may be conducted in the future but are much more time- and labor-intensive, requiring at least a year to screen animals; in addition, the small number of primates usually included in a study is small, thereby limiting the statistical power. Oral and intranasal routes of immunization have been tested and compared in mice and appear to be relatively ineffective.

Relying to another question, Dr. Berzovsky noted that the lack of an effect in the acute infection phase in macaques may have resulted from insufficient vaccine levels and poor CTL expansion. Modifications in study design and vaccine construct may overcome this problem. In contrast with monkeys, mice showed an immediate protective effect, as described.

Novel broadly cross-reactive HIV neutralizing antibodies and conserved structure of its envelope glycoproteins (Mitko Dimitrov, Ph.D.)

Dr. Dimitrov's research focuses on three primary areas: the characterization of co-receptors at the point of HIV entry, the identification and characterization of conserved envelope structures, and the development of HIV entry inhibitors and vaccines. He reported on recent discoveries, including the identification and characterization of new human monoclonal antibodies and the identification of novel gp41 conserved structures.

The development of an effective vaccine against HIV infection would be significant to public health nationally and internationally. More that 40 million people worldwide are infected with the virus, and current medical therapies can carry serious side effects, including drug resistance.

One model of HIV entry involves interactions between gp120, CD4, and co-receptor molecules; the co-receptor—gp120 interaction transmits the signal to gp41 to mediate major conformational changes and entry of HIV into the cell via the exposure of a fusion peptide. This mechanism of entry and the transient epitopes appears to be highly conserved. Ongoing research is investigating whether CD4 and the co-receptors can induce conserved epitopes on the HIV envelope and whether these epitopes can be targets for broadly cross-reactive HIV-neutralizing antibodies. Approximately 2 years ago, published studies reported that a fusion-competent immunogen could serve as an intermediate env to elicit broadly neutralizing antibodies, which, in turn, block entry of HIV into the cell. Researchers have not been able to repeat these experiments, however.

Using a different strategy to try to answer the same questions, Dr. Dimitrov and his colleagues prepared purified, immobilized env(gp120)-CD4-CCR5 complexes (in a 1:1:1 molar ratio) as immunogens; the complexes also have been used for screening of phage display libraries. These molecules interact with each other, and several steps are taken to ensure that the complex includes a soluble form of CCR5 with an appropriate conformation, that is, a conformation that allow interaction between CCR5 and gp120.

The complex was then used to screen for human libraries, with Dr Dennis Burton serving as a primary collaborator on the project. The sequence of the CDR3 heavy chain of the cross-reactive HIV-1 neutralizing human monoclonal antibodies (i.e., "X5") is very long but comparable with the sequence for b12, an antibody against the anti-CD4 binding site. The crystall structure of the CDR3 chain of X5 is somewhat similar to that of b12 and 17b. The primary property of this novel antibody is that it binds to gp120; binding to gp120 is enhanced markedly in the presence of sCD4 and CCR5. Thus, the epitope is outside the CD4 and the CCR5 binding sites. X5 is a high-affinity antibody that binds to env with deleted or nonfunctional variable loops and several primary isolates. Mapping studies indicate that X5 epitope overlaps partially with the 17b epitope.

Further investigations examined the ability of X5 to neutralize primary HIV-1 isolates in comparison with other neutralizing antibodies. Results of these experiments demonstrated similar overall responses in FabX5 and IgGb12. Overall, Dr. Dimitrov stated, the efficacy of the two antibodies was comparable given a range of conditions.

Briefly, X5 blocks HIV by binding to a CD4-induced epitope. X5 is also highly effective against sCD4-induced cell fusion for a number of X4 and X5 envs; inhibition is complete at concentrations as low as 0.1 μg/mL. Preliminary experiments suggest that X5 acts by blocking a post-receptor binding event. These findings may prove instructive in developing a vaccine to protect against HIV infection.

In conclusion, X5 is a human monoclonal antibody that was identified by screening a phage display library. It has a long, relatively hydrophobic fingerlike heavy chain CDR3. It binds to a variety of primary isolates and to variable loop mutants and is CD4-dependent. The epitope of X5 may play a role in the transduction in the activation signal from gp120 to gp41, thereby initiating major conformational changes that lead to fusion. Its binding site may be proximate to the gp41 binding site on gp120. X5 also inhibits the infection of PMBCs by primary HIV-1 isolates. The mechanism of inhibition appears to involve blocking of post-CD4 co-receptor binding events. The identification of X5 suggests the existence of conserved epitopes on gp120 that are induced by receptors outside the co-receptor binding site. These epitopes can be recognized by the immune system in infected individuals and serve as targets for other neutralizing antibodies and other HIV entry inhibitors.

Another series of experiments developed a new env glycoprotein that is stabilized by flexible linkers. This glycoprotein is a potent entry inhibitor. Fusion peptide usually is exposed when gp41 and gp120 are separated following specific post-binding conformational changes. Gp41 is noncovalently associated with gp120, making their binding unstable; however, connecting these two proteins using flexible linkers stabilizes this union. Several linked proteins were constructed and expressed successfully. One linked product that was predominantly gp140 (with a low level of gp120) was able to bind to co-receptor complexes, including sCD4 and cells that express CCR5. Binding is specific and the complex contains the necessary conformational epitopes.

While conducting these experiments, Dr. Dimitrov and his colleagues observed that certain antibodies had a higher and stronger binding affinity for the tethered constructs than for the native (uncleaved) compounds. The tethered protein was examined further in binding inhibition studies. Results of these studies suggested that the tethered construct possessed potent inhibitory ability. The construct appears to act through a gp41-related mechanism to inhibit membrane fusion. Preliminary results in mice indicate that the linked protein stimulates broadly cross-reactive neutralizing antibodies; however, further investigations are needed to draw more definitive conclusions.

In response to a question about the potency of the fusion epitopes, Dr. Dimitrov stated that he and his colleagues have tested other linked glycoproteins (e.g., gp140-26) that also bind to env. Some of these tethered proteins have been highly effective in their native environment. An attendee suggested that the increased effectiveness and high affinity of these tethered compounds may be due to increased gp120 binding to CD4, which would, in turn, produce a synergistic response.

Suppression and activation of KSHV replication by natural products (Denise Whitby, Ph.D.)

One of Dr. Whitby's key research interests is the role of environmental factors in Kaposi's sarcoma-associated herpes virus (KSHV) transmission and disease. Four different types of Kaposi's sarcoma (KS) have been identified and characterized. One form of the disease ("classic KS") occurs primarily in elderly men of Mediterranean and Jewish heritage and is generally benign. African-endemic KS also is found mostly in men; it is a more aggressive form of the disease than classic KS and can be fatal. Another form of the disease is found in iatrogenically immunosuppressed transplant recipients; it is relatively aggressive and occurs soon after transplantation. The disease is resolved if immunosuppression is removed. The fourth type of KS is AIDS-associated KS, which became a more predominant form of the disease following the initial AIDS epidemic in young homosexual men in the United States. AIDS-associated KS is an aggressive disease and can be fatal.

The KSHV envelope has several different spikes, in contrast with HIV, which has only one spike. Inside a proteinacious layer known as the tegament is the viral capsid, which contains the genome. KSHV is a gamma herpes virus; the herpes virus family also includes alpha and beta classifications. The human virus most closely related to KSHV is EBV. KSHV has a long viral genome that includes 80 to 90 open reading frames (ORFs). The genome structure has several regions that are highly conserved across all herpes viruses in addition to regions that are unique to KSHV. Theunique genes include some, such as apoptosis inhibitors, cylcin, and chemokines, that were captured from the human genome and are thought to be critical in pathogenesis.

Despite the array of genes present in KSHV, the virus is difficult to grow in culture. Researchers have exploited the virus's ability to cause primary effusion lymphoma to develop and establish lymphoma cell lines that can be latently infected with KSHV. The virus can also infect 293 cells, but full replication of the virus does not occur. Other systems and cells that have some susceptibility to KSHV infection include primary endothelial cultures and bone marrow stem cells; however, working with these systems poses several challenges.

Prior to the identification of KSHV and before the AIDS epidemic, an infectious agent was suspected to be the cause of KS primarily because of the disease's distinctive geographical distribution and its association with immunosuppression. The distribution of KS in AIDS patients suggested that the putative agent may be sexually transmitted. In 1994, Chang and Moore published their discovery of sequences from KSHV in a KS lesion. Soon thereafter, researchers sought to determine whether this was an accidental infection in KS or the cause of KS. To answer this question, Dr. Whitby and her colleagues examined biopsies from all forms of KS for the presence of KSHV. Cumulative data from a number of studies confirmed that KSHV did indeed occur in the four types of the disease. Additional early work by Dr. Whitby involved analyzing PMBCs of HIV-infected patients with and without KS for KSHV; using nested PCR, she found the virus more often in patients with KS than without KS. However, a 5-year follow up of patients initially without KS demonstrated that more than half of those in whom KSHV was detected eventually developed KS. In contrast, only 9 percent of those who did not have detectable virus at the initial screen did not develop the disease. These findings contributed further to the hypothesis that KSHV was the etiologic agent for KS.

Additional evidence was sought using serological assays. As Dr. Whitby explained, detection of antibodies also correlates with KS risk. KSHV can be detected in the spindle cell (i.e., the tumor cells of KS). However, experiments using *in vitro* cell cultures and animal models have failed to produce definitive data thus far. Early serological assays for KS employed immunofluorescence forthe latent nuclear assay (LANA),; more recently, researchers have used recombinant protein or peptide ELISAs (i.e., ORFs 73, 63). Concordance between these assays have been poor overall, in large part because of biological differences in how individuals react to the virus.

A solid body of epidemiologic evidence for KSHV exists. In most of the world, the incidence of KS is very low. However, clusters of populations with the disease are found in Africa and around the Mediterranean. Prevalence of KS in healthy populations closely mirrors the incidence of the disease, as does the distribution of antibodies to KSHV from asymptomatic, healthy blood donors. Work published in 1990 showed that the distribution of KS in AIDS patients varied by risk HIV factor. For example, persons who have acquired HIV parenterally rarely developed KS, whereas those who became infected sexually developed the disease more often. Further investigation indicated that these differences were due to differences in prevalence of the virus. The incidence of KS before the AIDS epidemic shows that Africa was the epicenter for the disease. The distribution of KS incidence in Africa has changed since the AIDS era began. In Uganda, KS is now the most common cancer reported, accounting for more than 50 percent of all adult male malignancies. The incidence of childhood KS, even is HIV-negative children, is on the rise, suggesting that the prevalence of the virus may be changing. In South Africa, an estimated 8,000 new cases of KS are expected each year in the background of the HIV/AIDS epidemic. In West Africa, there is a low rate of KS but a high rate of KSHV; the reason for this seeming disparity is not clear, however. Results of additional studies suggest that HIV/KSHV coinfected patients have a 50 percent risk of developing KS. Thus, the significant numbers of HIV cases in Africa coupled with the increasing prevalence of KSHV signal a major public health problem.

KSHV is an ancient virus that is believed to have co-evolved with the human species. The prevalence of KSHV varies geographically and closely matches the incidence of KS. Antibody titre and viral load in PMBCs in KS patients and healthy adults also vary geographically. Thus, the geographical variations in the incidence of KS can be explained in large part by differences in the prevalence of the virus. However, the wide variation in the prevalence of the virus cannot be explained as readily. Data indicate that the prevalence of KSHV is high in persons born in Africa but not in those whose parents were born in Africa. Similar findings are reported for persons of African descent born in the Caribbean.

Host and environmental co-factors that enhance virus transmission may play an integral role in KS pathogenesis. The NCI "oncoweed" study conducted by Dr. Whitby and her colleagues seeks to identify potential environmental co-factors that may be involved in HHV-8 (i.e., human herpesvirus 8, KSHV) transmission and disease. Most studies that have examined co-factors have compared persons with the disease versus asymptomatic controls. Thus, most of the suspected co-factors are most likely co-factors for infection instead of for disease. The primary hypothesis for the NCI study is that environmental co-factors reactivate HHV-8 *in vivo*, which leads to increased viral shedding and increased transmission of the virus, thereby leading to a higher prevalence, higher viral load, higher antibody titre, and greater risk of KS in areas where these environmental co-factors exist.

To test this hypothesis, Dr. Whitby and her colleagues first set up an *in vitro* screen to identify environmental agents that could reactivate HHV-8. They collaborated with investigators in the NCI's Natural Products Branch (NPB) on this arm of the study. The researchers accessed the NPB's repository, which includes more than 50,000 plant samples from tropical and subtropical countries in addition to marine invertebrates and algae. The NBP scientists produce organic and aqueous extracts from these items for use in anti-tumor and anti-microbial screens. In contrast, Dr. Whitby's team sought to identify products that would activate HHV-8 and induce carcinogenesis.

The method used in the oncoweed activation screen involved incubating 50-μg irradiated aqueous extracts in 96-well trays with latently infected cells (primary fusion lymphoma cells) for 3 days; ran a 96-well DNA extraction; and conducted two real-time PCR analyses (i.e., one for *erv*3, a cell quantification marker; one for KSHV). Viral load (per million cells) was calculated using these measures; extracts causing an increase in viral load were examined further. Because approximately 5 percent of the controls cells, which were activated by sodium butyrate, underwent lysis, extracts suppressing this process were considered potential candidates for additional study as antiviral or anti-gamma-herpesvirus agents.

Dr. Whitby and her colleagues screened 4,841 natural products from 38 countries; most were land plants from Africa. A total of 184 extracts activated HHV-8 as well as or better than sodium butyrate, more than 500 inhibited the virus, and 2,965 were inactive in the test system. These data indicated that KSHV can be reactivated *in vitro* using natural products. Results sorted by continent suggested that the origin of the plant or product was not related to the product's ability to activate the virus. However, many of the products that were effective were from tropical regions that also have a high prevalence of KSHV (e.g., Southeast Asia, South America). A trend toward increased potency to reactivate the virus *in vitro* was observed for products from Africa in comparison with other active products. Multivariate logistic regression analysis identified three families, each containing at least 25 natural extracts, as showing the greatest ability to activate HHV-8. Plans to characterize the top 28 activators further are underway.

Dr. Whitby concluded her presentation by noting that the findings of the oncoweed screen demonstrated that natural products are capable of reactivating HHV-8 *in vitro*. However, no one oncoweed explains the epidemiology of KS. The activators identified included medicinal plants, food plants, and plants used for the construction of fibers (e.g., for basket making). Thus, the activators are derived from plants with common human uses, thereby supporting the hypothesis that environmental factors may influence the activation of KSHV in the body. The oncoweed that serves as a co-factor in this process may vary regionally.

In response to a comment from one attendee, Dr. Whitby agreed that a next step in this investigation involves interviewing residents in the regions with the highest prevalence of virus and the highest incidence of disease. However, she noted, prior to the screen, the researchers did not have a clear idea as to which environmental factors may affect viral transmission or activation. The results of the *in vitro* screen should facilitate the development of questions for interview process, in addition to identifying the persons most likely to be exposed to these plants, products, or agents. Follow-up experiments on the top 28 activators will include characterizing mRNA and the pathway(s) of reactivation.

Dr. Whitby confirmed that different subtypes of KSHV do exist and that the subtype variations are largely geographical. However, data thus far show no association between virus subtype and disease risk. The lab is very interested in KSHV genotyping, she added. Researchers have little information on the natural history of the virus over time. Evidence suggests that the virus is poorly transmissible. For example, less than 50 percent of the spouses of elderly KS patients in Italy became infected with the virus or showed evidence of infection over periods of up to 40 years.

Regarding inhibition of the virus in the activation screen, Dr. Whitby noted that the cultures were never completely latent, suggesting that the screen was not highly robust for inhibition.

In response to another question, Dr. Whitby stated that she is very interested in studying cytokines, cytokine activation, cytokine genotype, and the effect of secondary infections on KSHV transmission, activation, and disease development and progression. An Epi Branch graduate student, Beth Brown, will be working with Dr. Goedert in examining cytokine genotype in samples from a "classic KS" case-control study conducted in Sicily.

Evolution of drug resistant HIV in vivo (Frank Maldarelli, M.D.)

Dr. Maldarelli is investigating the evolution of drug-resistant HIV in various populations, including drug-naïve and drug-experienced HIV-infected persons. A primary goal of studying drug-naïve HIV-infected persons is to determine the diversity of the genetic structure and the population characteristics of HIV in this group. *In vitro* mutation rates provide estimates of how drug resistance evolves, but population-based studies may present a more accurate *in vivo* picture of the virus. The size of the population studied drives the type of analysis conducted. For example, deterministic models may be used in conjunction with large populations, whereas stochastic models may be more appropriate for studies of small populations. The use of these models on HIV-infected populations in which the virus undergoes replication (i.e., the "effective population", Ne) should assist in predicting how drug resistance arises, predicting trends in the HIV epidemic, and identifying genetic variations post-infection.

Further studies will help characterize viral evolution within human populations as large predictable viral swarms, quasi-species, neutral mutations with drift, or small populations heavily influenced by random variation. Replication characteristics that facilitate the study of the genetic evolution of HIV include knowledge of the generation time of HIV (i.e., approximately 1 day) and the HIV mutation rate (i.e., approximately 0.5 and 5 x 10^{-5} mutations per cycle, with some contribution from RNA *pol*II). However, despite this cadre of relatively well-characterized factors, Ne remains uncertain. If the mutation rate is significantly larger than 1 (e.g., Ne >> 100,000 virions/individual), then a deterministic model is appropriate. If the mutation rate is significantly less than 1 (i.e., Ne << 100,000), then a stochastic model should be applied. Arguments supporting both models have been published.

The requirements for studying HIV population genetics in vivo include relatively frequent sampling and an intensive sequence analysis. Frequent monitoring over long periods of time is necessary to determine the overall structure and flow of the population and to detect the emergence of minor mutations. For example, to detect a mutation with a selective advantage of 1 percent over a nonmutated virus requires observation over approximately 100 replication cycles, or about 4 months. *In vivo* studies also need to evaluate whether infected patients represent a conglomeration of an isolated population or a relatively heterogeneous population. These studies should include extensive genetic analyses to obtain the genetic sequence of as many individual genomes of HIV as possible. The region of greatest interest is HIV *gag/pol* region associated with drug resistance, including HIV protease and the the first 1,200 nucleotides of reverse transcriptase. The approach used to obtain this information involves an endpoint dilution-PCR amplification strategy.

The protocol will enroll drug-naïve HIV-infected patients into a longitudinal observational study.

Participants will be evaluated daily for 10 days, weekly for 4 months, and monthly for 18 months. Patients who become eligible for treatment during the course of the study (i.e., CD4 count < 350, viral load >50,000) will be placed on therapies as needed and will continue to be monitored according to the study design. Eighteen persons have been enrolled into the study thus far; about five have started on antiretrovirals.

Dr. Maldarelli described one participant who had been HIV negative through January 2000. The person reported having an unsafe sexual event on May 29, 2000, which was followed by an acute viral episode in mid-June 2000, at which time he was admitted to the hospital. An ELISA done during the patient's hospital stay was positive, but a Western blot was negative. and the viral load was approximately 500,000 copies/mL. At the time of enrollment into the NIH study (7/6/00), a repeat ELISA and Western blot were both positive. Following initiation of antiretroviral therapy, the patient's viral load dropped to less than 50 copies/mL, where it remained for 18 months. Specimens collected from the participantrevealed that this person had a highly monomorphic virus population; analysis of 24 sequences in a single plasma sample, revealed 17/24 amplicons were identical and a total of eight changes observed overall. Another participant described by Dr. Maldarelli had been diagnosed as being HIV positive 10 years prior to study enrollment. The patient, whose CD4 count had decreased slowly from about 500 to 350 over the 10-year period, had consistently refused anti-HIV treatments. In January 2001, he developed the flu, and his viral load increased from approximately 19,000 copies/mL to approximately 136,000 copies/mL. His CD4 count remained stable at 350. Topological tree analysis of about 1,500 nucleotides over 9 consecutive days indicated a relatively even distribution of genetic sequences on a day-to-day basis. Trees were similar while the patient's viral load was elevated during his illness and when the viral load returned to its usual level following his recovery. Thus, this patient's HIV population had little variation. [At this point, an attendee commented that it would be interesting to examine the patient's topological tree using specimens collected prior to his developing the flu and to determine whether the reduction in viral load upon recovery actually represented a collapse of an expansion.] Dr. Maldarelli cited similar findings in another drug-naïve patient who had been HIV positive for about 1.5 years.

In response to inquiries about the statistical methods applied to the study, Dr. Maldarelli stated that using a standard number of randomly distributed samples and families, it would be statistically possible to detect differences in specific branches within a family. However, the current study would require a much larger number of sequences and samples to ensure sufficient power to detect such differences. Overall, models using these methods indicate that if genetic changes are occurring, they are proceeding at a very slow rate.

Dr. Maldarelli reported that preliminary findings suggest that *pol* allele frequencies samples from the second participant are highly conserved; of 1,497 nt sequences, 1,292 positions were identical in 168 sequences. Regarding informative sites, analyses indicated 205 nonidentical sites; 72 sites had a frequency of a minor allele greater than 2 percent; and 24 sites had a frequency of a minor variant of at least 10 percent. Two-thirds of the 24 sites (16/24) were synonymous changes. During a 7-month follow-up monitoring period, no new alleles were detected, no alleles were extinguished, and no allele frequency differences were detected. Running a heterogenicity Chi square on the entire sample suggested that the sample was uniform. Tests of variations in HIV RT indicated that none of the variations identified were at sites associated with drug resistance. Variations did not occur at typical CTL epitopes reported for the patient's HLA subtype

Results of rudimentary linkage analysis indicated that alleles in unlinked sites have resorted by recombination, whereas alleles in linked sites have not resorted. >From these data, Dr. Maldarelli and his colleagues predicted the expected allele frequencies and compared the expected with observed frequenciesThe general conclusion of this analysis on the 24 sites was that neighboring loci were usually but not universally linked. Histogram distribution showed linkage occurred most frequently between loci 20 nt's apart; loci 21 to 40 nt's apart were often linked polymorphisms; and loci greater than 40 nt's apart were increasingly less likely to be linked. A polymorphism at position 499, which was unlinked to alleles just three nucleotides both upstream and downstream, may represent the emergence of a new allele. The explanation for the presence of linkage disequilibrium for alleles separated by some distance remains uncertain although RNA structural considerations may be involved. Further analysis will attempt to answer these questions.

In closing his presentation, Dr. Maldarelli commented that genetic variation in RT and protease is very difficult to detect in acutely infected patients. However, there is evidence of frequent polymorphism and recombination in chronically infected patients. Drastic changes were not observed over time or with the administration of antiretrovirals, suggesting a large effective population size.

Adjournment

The meeting was adjourned at approximately 4:00 p.m.