SEVENTH NIKOLAS SYMPOSIUM
May 10-13, 1996

Friday May 10
Introduction

Dr. Jon Pritchard presented a sketch of **clinical aspects** of LCH, epidemiology, long-term consequences of disease and a list of prior symposium topics. It was noted that extensive epidemiologic studies have not been done for LCH and that because no national or international registry exists, definitive information is lacking. Although overall mortality is only about 10%, up to 50% of survivors will experience significant late effects. These late effects include diabetes insipidus, CNS disease including cerebellar involvement, liver fibrosis, pulmonary fibrosis, growth retardation, hearing deficits and dentition problems. The association of LCH with malignancies was discussed.

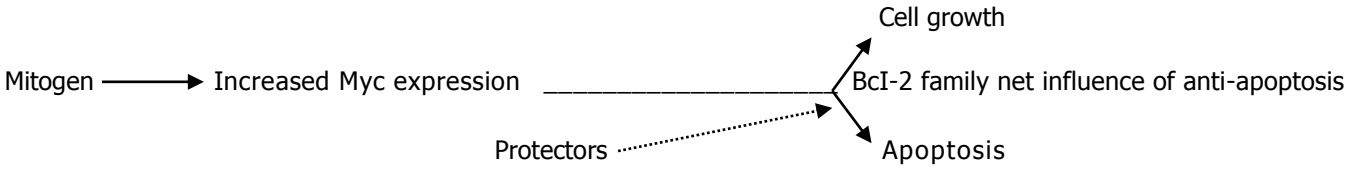
Dr. Ron Jaffe presented the pathology of LCH by pointing out diagnostic criteria and noting cases that deviated from these requirements. He suggested that attention be given to the latter. Preliminary data was presented suggesting that CD1 a positivity decreased with increasing grade of histological morphology, i.e., from localized eosinophilic granuloma to true malignant disease. In addition, immunohistochemical analysis of cyclin expression demonstrated that between 2-30% of cells within LCH lesions are actively proliferating.

Dr. Blaise Favara briefly commented that as more cases are studied it is apparent that the composition of LCH lesions varies; there is heterogeneity between different lesions in the same patient and among lesions from different cases.

Saturday May 11
Apoptosis 1

The integrated control of cell proliferation and cell viability was presented by ***Dr. Nicola McCarthy.*** The role of c-myc in cell growth, differentiation and apoptosis has been extensively studied but various aspects remain controversial. The data presented centered on work in a rat fibroblast system. Constitutive expression of c-myc in these cells induces apoptosis. This effect can be abrogated by exposure of the cells to insulin-like growth factors I and II (IGF-1 IGF-ll), as well as platelet-derived growth factor (PDGF). Although epidermal growth factor (EGF) is mitogenic for these rat fibroblasts, it does not induce apoptosis, thus separating mitogenic from survival effects.

It was further shown that c-myc expression induces cell death through an action during S phase of the cell cycle. Thus, in some systems, cells may continue to grow until survival factors are depleted following which programmed cell death is initiated thereby curbing the proliferating population.



Further downstream in the apoptotic pathway are the terminal execution molecules such as the cysteine proteases exemplified by the ICE (lnterleukin-1 Converting Enzyme) proteases. Several ways to inhibit these proteases have been described and work on one inhibitor, ZVAD, was presented. Of interest, ZVAD appears to be able to modulate the time to cell death, but does not ultimately prevent the process. Either such inhibition is not complete or there are other pathways which can be initiated to complete the apoptotic program. Cytoplasmic blebbing was demonstrated as an early sign of cell death by apoptosis. It probably anticipates nuclear DNA changes.

Thus, although still unclear how survival factors and c-myc expression are mechanistically interwoven and responsible for inducing an apoptotic program, such pathways may prove to be important in the survival of specific cell types both during health and disease.

The role of p53 and its downstream targets on apoptosis was presented by ***Dr. Peter Hall.*** Recent Work from several investigations have shown the importance of the tumor suppressor gene in the development of cancer as well as in cell cycle arrest in response to genotoxic damaging agents. Such observations have resulted in p53 being viewed as what Lane described as "the guardian of the genome." Once DNA is damaged p53 expression is increased leading to apoptosis and thus discarding of the cell bearing the damaged DNA.

Less work has been done on the expression and function of p53 in vivo following exposure to genotoxic agents. Using rodent model systems, work was presented on the expression of p53 in response to radiation versus radiation and cytosine arabinoside in the gastrointestinal tract, an organ particularly sensitive to such genotoxic agents. Interestingly, there was a profound heterogeneity in both apoptosis as well as p53 expression in the intestine. For example, in the small intestine, an early effect was increased p53 in the stem cell zone of the intestinal crypts, while in the large intestine, a late effect of increased p53 expression was observed in a crypt transit zone. Furthermore, following radiation exposure, several different cellular responses were demonstrated. These included cells with 1) increased p53 expression undergoing apoptosis, 2) increased P53 expression not undergoing apoptosis and 3) no detectable p53 expression and no evidence for apoptosis.

There are tissue-characteristic patterns of p53 expression:

p53 expression with apoptosis - Thymus, gut, spleen, bone marrow, choroid plexus

p53 expression with little apoptosis - Adrenal, kidney, bone, myocardium, smooth muscle

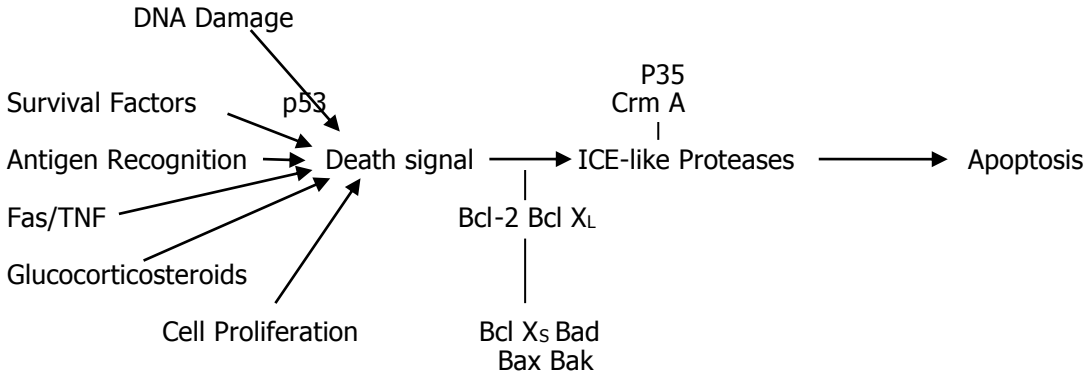
p53 expression with no apoptosis - Liver, skeletal muscle, brain.

This type of heterogeneity of response to genotoxic damage appears to develop during the later stages of embryogenesis. Of further interest is the recently discovered embryopathy observed in p53 -/- "knockout" mice characterized by neural tube defects and an excess of male pups.

Thus, 053 plays important roles as a tumor suppressor gene, a protective cell cycle regulator in response to genotoxic agents as well as having important developmental functions. How does p53 do all this? Part of the answer to this question comes from the identification of target genes regulated by p53. The GADD45 gene encodes a 21-23 kilodalton protein that is also induced by genotoxic agents as is the p21 protein. Data was presented showing that these two proteins associate with each other as well as with cyclin D and PCNA, thereby impacting upon the regulation of the cell cycle. The role of p53 during development, apoptosis and in cancer pathogenesis is clearly of great importance, but the precise mechanisms by which its effects are executed remain still largely undiscovered.

One of the Artemis Fellowship Award Recipients was ***Dr. Michael Weintraub,*** who presented a study on p53 expression in LCH: P53is usually not detectable by immunohistochemistry in normal tissues, so the finding of subP4ulatiOns of LCH Cells staining positive for p53 is noteworthy. Mutation causes p53 accumulation and subsequently a positive reaction. False positivity is possible. Each of the 10 LCH lesions studied were shown to have some positive staining for p53 with the numbers of positive cells varying between a minority and upwards of 90%. Immunohistochemistry for mdm2 was negative ruling out evidence of p53 bound to cell protein. DNA analysis of exons 4 through 11 (the major sites for p53 mutations) was performed and no mutations were found (PCR/SSCP method). Thus, the possibilities of increased expression and/or p53 protein stabilization in LCH lesions was proposed. Increased expression could be possibly induced through the action of certain cytokines such as TNF-alpha while p53 stabilization is known to occur following interaction with certain viral encoded proteins such SV4O T antigen and adenovirus Ela. This last point brought up a 'discussion of the possibility of an as yet unidentified viral etiology to LCH. The use of the TUNNEL technique of immunohistochemicalstaining for apoptotic cells in LCH lesions was discussed and preliminary data indicated that lesions contain between 5 to 10% positive cells by this method. However, it was also pointed out that the TUNNEL method does not detect all DNA strand breaks and that morphology is probably still one of the best assays for enumerating apoptosis in tissue sections. This controversial topic came up on subsequent days of the meeting.

Dr. Gabrielle Nunez presented work on **the regulation of apoptotic death by the Bcl-2 family** of proteins. An extensive overview of the Bcl-2 family of apoptosis regulatory proteins was presented including those proteins that block apoptosis as well as those that facilitate the process. A relatively delicate balance of expression and interaction within the cells of these blockers and facilitators is fundamental to the regulation of programmed cell death. Some members of the BcL-2 family block and some facilitate apoptosis. It is the net effect that determines outcome. The following schematic summarizes some of these interactions:



Work was presented on transgenic mice expressing Bcl X_L and or Bcl-2 demonstrating the critical role of these proteins in B lymphocyte development and survival. Of further interest were experiments showing that Bcl X_L transgenic mice develop autoimmune disease which was still partially dependent on T lymphocyte help.

The clever use of positive regulators of apoptosis for potential therapeutic interventions was presented. By linking the Bcl X_S gene, which encodes a facilitator of apoptosis to an adenovirus vector with an RSV (respiratory syncytial virus) promoter, it was shown that bone marrow could be purged of tumor cells (breast cancer cells) and that normal hematopoietic progenitors were not adversely affected. Such an approach might also provide a means for non-chemotherapeutic based therapy in LCH.

Fas pathways and apoptosis was introduced by ***Dr. Abul Abbas.*** Fas (CD95, APO-1) belongs to a large family of proteins including the TNF-receptor/ligand family and the B cell antigen CD40 that play important roles in cell proliferation and cell death. Fas is mostly expressed on the surface of T lymphocytes as a trimeric receptor of the Fas ligand. When the Fas receptor is stimulated it induces a series of steps through a not yet identified number of signal transduction steps which lead to the activation of ICE-like proteases and its facilitation of apoptosis. The Lpr and Gld mouse represent natural mutants in the Fas receptor and Fas ligand respectively. These mice develop CD4 helper cell dependent autoimmune disease as well as a lymphoproliferative or (or more accurately a lymphoaccumulative) disorder as a result of the defects in the Fas pathway of apoptosis. Several patients with autoimmune disease, lymphadenopathy and circulating CD4-/CD8- T lymphocytes have been described with mutations in the so-called "death domain" of the Fas receptor.

Data were presented for CD4 positive lymphocytes showing that naive T lymphocytes proliferate and differentiate when they receive antigen stimulation plus a costimulatory signal or IL-2. When a naïve T lymphocyte receives an inadequate antigen stimulus, programmed cell death is induced, but this can be prevented by exposure to IL-2 or a costimulatory signal. However, in already activated T lymphocytes, exposure to antigen and IL-2 results in programmed cell death. In other words Il 2 (a "death factor") enhances this pathway in activated T cells. This pathway works only in peripheral (post-thymic) T cells when T cells are activated and there is co-expression of Fas and Fas ligand. When the T cells are activated the Fas/Fas ligand combine. The Fas tail functions like a protease converting ICE and including apoptosis. Apoptosis results independent of Bcl-2 family.

Of interest in this regard were data presented from CD25 (i.e., the alpha chain of the IL-2 receptor) homozygous "knockout' mice which develop autoimmune disease and too many T lymphocytes. The following table is a summary of these results:

	CD40 Dependent Apoptosis	
Induced by:	Inadequate Antigen Stimulation	Activation Induced Cell Death
1L-2	Protects	Enhances
Costimulation	Protects	Does Not Protect
Fas	None	Obligatory
Bcl X_L	Protects	None

Thus, IL-2 may have an important role to play in not just activation and expansion of T cells, but also may function as a type of programmed cell death inducer for already activated T cells. Such conclusions may have significance for LCH based upon data presented on cytokine expression in lesions (see later).

Apoptosis 1

Examples of molecular determinants of intrinsic chemosensitivity in human tumors was presented by ***Dr. Christine Chresta***. It is now relatively well established that chemotherapy-induced killing of tumor cells is usually the result of triggering apoptosis. Thus, the resistance of tumor cells to chemotherapy may result in part from altered regulation of apoptosis. The role of molecular pathways controlling apoptosis therefore has important implications for the success or failure of chemotherapy-based treatment of cancer patients.

Comparison of several apoptosis regulatory proteins was made in cell lines from chemotherapy sensitive testicular germ cell tumors and drug-resistant transitional cell cancer of the bladder. The drug accumulation in the cell lines being compared was equivalent, thus ruling out some mechanisms of drug resistance such as increased expression of the multidrug resistance P-glycoprotein. Of particular note was that the apoptosis facilitator protein, Bax, was shown to be expressed in the germ cell tumor lines but not in the drug-resistant bladder tumor cell lines. Conversely, the apoptosis inhibitor, Bcl-2, was expressed in the bladder cell tumor lines but not in the germ cell tumor lines. Differential expression of apoptosis regulators were therefore suggested to play an important role in chemotherapy resistance and sensitivity. Both tumors suffered DNA damage secondary to VP 16 that lead to apoptosis.

Further work including gene transfer and expression studies of these different apoptosis regulators as well as transgene and homologous knockout experiments will be needed to better define their precise contributions to different tumor type chemotherapeutic resistance patterns. However, such studies may be worth applying to LCH lesions in terms of helping to define possible mechanisms of drug resistance and therefore alternative strategies for improving treatment

Dr. Klaus-Michael Debatin introduced **the role of CD95 in growth control of lymphohaemopoietic cells** by presenting the rationale and development of apoptosis-inducing antibodies which led to the identification of APO-1 or Fas (CD95). This type of triggered cell death was contrasted with that of spontaneous cell death or death by default resulting from growth factor withdrawal. Activated T lymphocytes were shown to up-regulate Fas on their surfaces and subsequently demonstrate increased sensitivity to anti-Fas antibody induced apoptosis. In addition, several cytokines including 1L-3, G-CSF, GM-CSF and stem cell factor appear to increase CD95 expression on a variety of hematopoietic cells. Of particular interest were experiments demonstrating that in the drug sensitive human T leukemia cell line, CEM, exposure to doxorubicin induced the expression of CD95. In contrast, in a drug-resistant derivative of CEM, doxorubicin exposure does not increase CD95 expression. The CD95/CD95-ligand interaction serves as trigger. These experiments thus link the expression and function of Fas/CD95 to chemotherapy-induced programmed cell death. They also may offer another mechanism by which tumor cells become resistant to chemotherapy.

Cell death pathways are distinctive:

- Spontaneous death by default – ie, withdrawal of growth factors
- Triggered death - Via activation pathways - ie, TCR/CD3 in T cells
  APO-1 "death receptors"
- Steroid-induced cell death
- TNF/NGF receptor superfamily includes APO-1, Fas and CD95

Examples of apoptosis in hemopoietic disorders:

- Too much in leukemia/ lymphoma, lymphoproliferative disorders and auto immunity.
- Too little in AIDS, immunodeficiency, aplastic anemia and dyserythropoietic anemia.

In the final discussion Dr. Abul suggested culturing LCH cells with inhibitors of apoptosis.

Dr. Paul Smith gave in his presentation entitled: **Cellular targets for chemotherapy** a detailed overview of the different molecular targets within cells on which various chemotherapeutic drugs act. Both drug dose and time of exposure were shown to be critical determinants in the induction and level of programmed cell death. These variables are furthermore related to the stage in the cell cycle that a tumor cell is when exposed to a chemotherapeutic agent. The various "check points" in the cell cycle were identified and the roles of cyclins and other factors were noted. Factors in cell damage and in damage control are also relevant in that they determine if there is arrest in the cycle leading to death or to ultimately cell recovery. Thus, the exposure of tumor cells to specific chemotherapeutic agents results in the induction of apoptosis only at certain stages of the cell cycle.

Classes pf anti-cancer agenst are:

- Those that inflict direct DNA damage
- DNA topoisomerase inhibitors of 2 types: - those that disrupt DNA synthesis
  - those that disrupt mitotic spindles

Several important methodological points mere made in terms of studies on tumor cell sensitivity to chemotherapeutic drugs. Mechanisms of the development of cellular resistance to chemotherapy were noted and examples of interaction between agent and target cells of different kinds were shown. Such studies should be done using physiologically relevant concentrations of genotoxic drugs. Cell cycle should be measured and correlated with drug effects. Thirdly, methods which focused on measuring multiple parameters in individual cells are extremely important. For example, using multiparameter flow cytometry several important aspects of cellular physiology can be simultaneously measured such as DNA content and stage of the cell cycle, the level of surface or cell cycle regulatory proteins and whether a cell has entered the apoptotic pathway. It is upon these concepts and data that drug schedules and therapeutic strategies are developed. Such measurements are more likely to give definitive data on cell cycle checkpoints and entry into apoptosis than total population determinations of drug effects. These approaches will, however, be extremely difficult to accomplish on LCH specimens unless sufficient quantities of fresh materials, LCH cell lines become available or xenograft animal models are developed.

The evaluation of apoptosis as a possible prognostic factor in the treatment of leukemia was offered by ***Dr.*** **George** ***Tsangaris.*** Preliminary studies describing an approach to determining levels of apoptosis in bone marrow and pen peripheral blood specimens from patients with leukemia were presented. His group utilized the method of ethidium bromide staining and morphologic examination of fragmented nuclei. By this method both the morphologic type of the cell and whether it was undergoing apoptosis was possible to determine. Using this type of method, the degree of apoptosis can be relatively easily measured on routine bone marrow or peripheral blood samples at diagnosis and at various times following the initiation of therapy. He found that the presence of apoptosis was associated with bad prognosis. Perhaps the RES is failing to remove damaged leukemia cells. An additional sophistication of this approach might be to combine measurements of apoptosis with other markers (such as ploidy, cell cycle stage or surface antigens) simultaneously with the use of flow cytometry. Although the data presented could not definitively correlate response with the induction of apoptosis, this type of study may eventually prove important in light of the growing importance of the regulation of programmed cell death and chemotherapeutic sensitivity.

Sunday, May 12, 1996
Follow-up on Previous Research

An update of LCH Clinical Trials was given by ***Dr. Helmut Gadner***. There were several important aspects of this first international trial LCH I, including 1) the standardization of diagnostic criteria, 2) the standardization of response to therapy criteria, 3) a randomization of patients to one of two therapeutic arms, 4) sufficient numbers of patients to determine statistical- significance to different therapies and 5) the possibility of examining long-term outcome in a group of patients with the above characteristics of entry criteria and treatment. Initial results from this study have demonstrated an overall survival of 65%, a "reactivation-free" survival of 35% and an event free survival of approximately 30%. However, survival was about 85% for responders while it was only 35% for nonresponders, thus emphasizing the importance of initial response to therapy as a critical prognostic factor. The overall survival on Treatment Arm A (vinblastine plus steroids) versus Treatment Arm B (etoposide plus steroids) was the same at about 65%. In addition, reactivation-free survival on the two arms showed no significant difference and was about 45%. Of note, the incidence of diabetes insipidus on the LCH I trial was about 23%. From the data obtained in LCH I, a "low risk" group was identified and characterized as being greater than 2 years of age without involvement of liver, lung, spleen or the hematologic systems. There were no fatalities in this group of patients. Meta-analysis of the results from the DAL HX-83 Study Group (Medical and Pediatric Oncology, 23:72-80, 1994) compared to the data from LCH I was presented showing that the DAL therapeutic regimen (utilizing prednisone, VP-16, vinblastine, 6-mercaptopurine and methotrexate) resulted in a higher overall survival (85% versus 65% for LCH I) and a better reactivation-free survival (70% versus 35% for LCH l). These data thus have been used to design LCH II which will compare a two versus three drug induction regimen and two different maintenance regimens (6-MP, prednisone, vinblastine versus. 6-MP, prednisone, VP-16, vinblastine).

One of the Artemis Fellowship Award Recipients, ***Dr. Jim Whitmore*** described the occurrence (concurrence) of **LCH in monozygotic twins**. Is there a genetic basis for LCH?

Dr. Whitmore continued with a **study of prostaglandins in LCH** and presented an overview of the synthesis, physiology and functions of prostaglandins as well as their potential role in the pathophysiology of LCH. Emphasis was placed on the role of PGD2 in bone resorption. Work was presented by using a unique mass spectroscopic method for PGD2 developed at Vanderbilt University using urine as a source of the secreted prostaglandin. Normal ranges for adults have been established although normal values in pediatric age groups remain to be done. Data were presented on several children with LCH which tended to show high levels of PGD2 in their urine when they had active disease as opposed to when remission of disease status had been obtained. For example one of the above mentioned twins had levels 20X normal before therapy and 5X normal after therapy. A biology study to be attached to the LCH II clinical trial was proposed to study the level of PGD2 in LCH patients at diagnosis, during and following cessation of treatment. Such data along with sufficient information on normal, age-matched controls, might help to establish whether levels of urinary PGD2 is a marker for active disease or response to therapy.

Dr. Anthony Chu reviewed the status of **clonality studies** on LCH specimens as well as on normal dendritic cells. Langerhans cells migrate toward IL1, 1L3, IL8 and GM-CSF and cover 25% of the skin surface area. At a previous symposium Dr. Chu had presented preliminary data showing that dendritic cells from patches of normal skin might be clonal in origin. Data were presented from the skin of six patients undergoing breast reduction surgery. Dendritic cells and keratinocytes were isolated from these large patches of skin. The HUMARA (human androgen receptor assay) PCR based assay was used to determine clonality. The dendritic cells from two specimens were shown to be clonal in origin, in two other specimens the dendritic cells showed a skewed pattern demonstrating nonrandom but not clonal origin and in two other specimens the dendritic cells were polyclonal. In contrast, keratinocytes were shown to be polyclonal in origin in 4 samples and skewed in two other specimens. These results thus show that dendritic cells from relatively large patches of normal skin may be clonal in origin. These data further suggest that dendritic cells may arise from relatively limited numbers of hematopoietic precursors and then subsequently populate region of skin. Does this mean that the clonality reported for the Langerhans cells in LCH lesions is the result of the expansion of normal dendritic cells? Of interest is that every LCH lesion that has thus far been

reported has shown clonality when the HUMARA assay has been informative. In contrast, only about 30-40% of the normal samples of skin examined demonstrated dendritic cell clonality. Thus, the debate must continue as to what clonality means in LCH, although these intriguing data would suggest that Langerhans cells in LCH lesions are not merely the expansion of normal, clonal dendritic cells.

Dr. Eric Claaseen discussed cytokine production in Langerhans Cell Histiocytosis. Using carefully controlled immunohistochemical methods for the detection of specific cytokines, 15 frozen LCH were studied for the expression of a variety of cytokines. Dramatic amounts of cytokines in the LCH lesions were found: "Cytokine storm". The following cytokines were associated with Langerhans Cells, T cells and/or granulocytes in the indicated percent of lesions studied:

Cytokines	Specific Cell Types	Percentage positivity
IL1	Lcs	90%
IL2	T cells	90%*
IL3	T cells + gran	90%
IL4	T cells	100%
IL10	Lcs + gran	90%
GM-CSF	Lcs + T cells + gran	90%
TNF-alpha	T cells	100%
Interferon-gamma	Lcs + T cells	90%*

*These findings were challenged by Kannourakis who found none in his RT-PCR studies; neither did dr. Emile. DeGraaf had no data on these two cytokines.

Dendritic Cell Biology

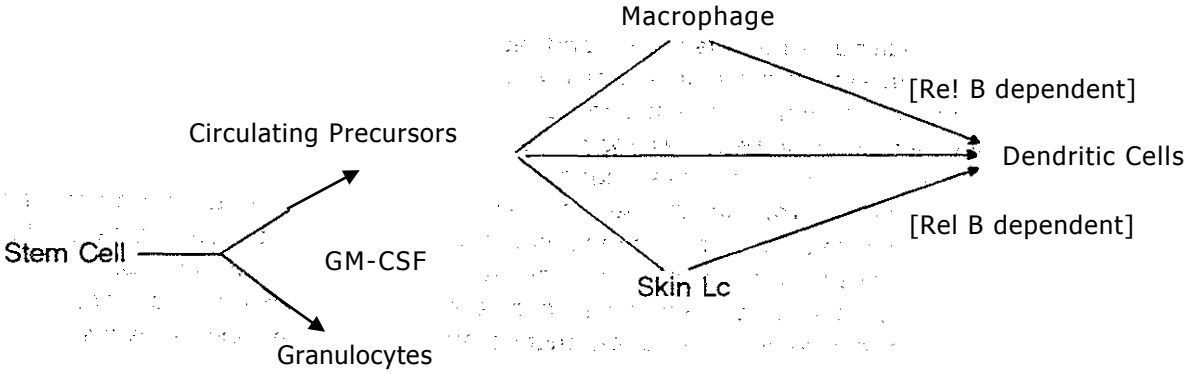
Dr. John Austin introduced **the dendritic cell development** in the mouse: [1] Dendritic cell precursors from bone marrow seed peripheral tissues where they undergo changes under the influence of GM-CSF and 1L4. [2] Immature peripheral dendritic cells have optimal endocytic properties. These cells, under the influence of TNF-alpha and CD40 ligand, become mature dendritic cells. [3] Cytokines cause movement from non-lymphoid to lymphoid tissues where they interact with T cells. Rel B has a role in the last two steps. The LCH cell may be the immature dendritic cell at step 2.

Rel B and dendritic cell development was presented by ***Dr. David Lo.*** The biology of dendritic and Langerhans cells remains an important area area of laboratory investigation in potentially improving our understanding of LCH and related disorders. Rel B is known to be a member of the NF-KB family of transcription factors involved in regulating immune responses and acute phase reactions. Rel B is expressed in the T cell zone of mouse spleen, in medullary thymus in epithelial cells with dendritic cell features. Rel B is not expressed in dendritic cell precursors, tissue macrophages, monocytes or Langerhans cells. However, when these precursors are exposed to differentiation cytokines or LPS, maturation into mature dendritic cells or activation of macrophages occurs. Associated with these changes is an upregulation in the expression of Rel B.

Mice with both alleles of the Rel B gene disrupted are viable but demonstrate important phenotypic characteristics related to the dendritic cell lineage. There is a block in the differentiation of dendritic cells as well as decreased numbers of dendritic cells. A major phenotypic alteration observed in the Rel B homozygous :knockout" mice is the disruption of Peyer's patches or white pulp in the spleen, lymph nodes and thymic architecture since dendritic cells are necessary for organization of lymphoid tissues. In contrast, these mice show pulmonary, splenic and portal infiltrates consisting of granulocytes, macrophages and lymphocytes. High-endothelial venules play a key role too.

Other studies demonstrated that there was a defect in negative slection in the thymus in Rel B -/- mice. Through a series of elegant experiments involving the transfer of normal dendritic cells into Rel -/- mice,

it was shown that splenic architecture could be partially restored. This type of experiment demonstrated the critical importance of dendritic cells in both the organization and function of the immune system. Based upon these and other studies, a differentiation program illustrating stages at which Rel B dependent pathways might function was proposed, as shown below:



A major effort for the future will be to identify the downstream genes which Rel B controls. It will be of further interest to determine if any of these downstream Rel B targets might be involved in the pathogenesis of LCH. Do LCH cells express Rel B? What determines the dendritic cell - T cell interaction? probably not TCR or adhesion molecules.

The role of GM-CSF in the pathogenesis of LCH was presented by ***Dr. Jean Francois Emile,*** another of the Artemis Fellowship Award Recipients. For several years now it has been known that GM-CSF is a growth and differentiation factor for dendritic cells. This presentation focused on the expression of GMCSF and its receptor in LCH lesions as well as the surface antigens present on Langerhans cells in LCH. GM-CSF was expressed in all LCH lesions examined by immunohistochemistry on frozen tissue sections. In addition to expressing GM-CSF, Langerhans cells were also shown to express the alpha chain of the GM-CSF receptor, thus creating the possibility of an autocrine stimulatory loop. Serum GM-CSF is usually not detectable in normal individuals, but in 3 of 15 patients with diffuse and progressive LCH, GM-CSF levels were detectable in the serum.

Other antigens found to be present on Langerhans cells in LCH lesions included ICAM-1, CD68, CD24, CD4, B7-1 (CD8O), B7-2 (CD86) and placental alkaline phosphatase. E-cadherin was reported to be present on Langerhans cells in cases of isolated skin lesions but negative in cases with diffuse LCH. Dr. Jan H. de Graaf argued that they have never been able to demonstrate E-cadherin expression in lesions of LCH. Thus, although some controversy persists in the precise expression of antigenic markers on Langerhans cells in LCH lesions, it is mostly agreed that these lesional cells express a variety of "activation" antigens, but that they are very inefficient in antigen presentation and T lymphocyte stimulation. This latter point is somewhat surprising in light of their expression of both B7-1 and B7-2 costimulatory molecules.

Studies on a murine model of LCH was presented by ***Dr. George Kannourakis.*** He began with a discussion of the possible pathophysiologic mechanisms contributing to LCH including cytokine expression. Work from their group demonstrated expression of 1L-1, 1L-4, 1L-8, 1L-10, Transforming Growth Factor-β (TGF-β), TNF-alpha, GM-CSF and very high levels of Leukemia Inhibitory Factor (LIF). Although the importance of T cells in LCH lesions was emphasized, in contrast to the data presented by Dr. Eric Claasen, they do not detect any 1L-2 or IFN-γ. A cytokine profile characteristic of a TH_2 response was presented.

In terms of trying to better understand the immunologic response in LCH, data examining the use of T cell receptor V-beta classes were presented. Of particular note was the finding that a severe restriction to only V-beta 1 and/or 7 are observed in patients with disseminated, progressive and sometimes premorbid disease. No restriction in V-beta usage was observed in patients with more limited disease. This would be the only example of a disease state resulting in such profound V-beta restriction. Restriction appeared to be the result of deletion rather than expansion. Subsets not present are on T cells that are dying.

Dr. Kannourakis founds increased apoptosis using the TUNEL method. Caution in this regard was urged by Dr. Peter Hall. An hypothesis was presented that suggested that a non-conventional antigen might be

responsible for the activation and subsequent apoptosis of the majority of T cell V-beta subsets.

In an attempt to further understand the pathobiology of LCH, preliminary results of an animal model for LCH were presented. Cells from an LCH lesion were injected subcutaneously along with GM-CSF and TNF-α into a SCID (severe combined immunodeficient disease) mouse. Serial transfer of cells from the spleen or liver into other mice were said to result in granulomas. Approximately 3-4 months Later, one of the mice developed a thymoma with splenomegaly, nodular liver, big mesenteric nodes and thick gut wall. The mice form granulomas that include multinucleated giant cells and histiocytes. A thymic lymphoma developed in one case. A cell line was obtained from this murine T cell lymphoma. Reverse transcriptase activity was shown to be present in this lymphoma. In addition, electron microscopy showed budding virus demonstrating that a retrovirus was likely to be the etiologic agent in the development of this thymic derived lymphoma, so it was suggested that he found a new murine retrovirus.

It was further stated (no data shown) that the serum from LCH patients reacted with the murine lymphoma cell line. The hypothesis was made that an retrovirus endogenous to the cells in the LCH lesion recombined with a murine virus causing the T cell thymic derived lymphoma. Current experiments were being directed toward immunoprecipitation of membranes from the murine lymphoma cell line with LCH human serum as well as isolation and sequencing of the murine lymphoma cell line retrovirus.

A Summation and Discussion was mediated by *Dr. Abul Abbas.*

The expression and function of cytokines in LCH lesions has been mostly accepted as being at least in part contributory to the pathophysiology of LCH. IL-3 and GM-CSF along with several other cytokines noted above were mostly agreed upon as being expressed in LCH lesions. However, important controversy still exists over whether a few cytokines are expressed or not. Dr. Eric Claasen's presentation demonstrated that IL-2 and IFN-γ are both expressed in LCH lesions while Dr. George Kannourakis stated that in their studies they could find no evidence for either of these two cytokines. Further studies need to be done to help resolve this important controversy. In spite of the growing evidence for the expression of specific cytokines in LCH lesions, it is still unclear what the etiology of their increased expression is.

Equally enigmatic is the presence of activated T lymphocytes in LCH lesions, while the Langerhans cells present in LCH lesions appear incapable of activating lymphocytes, in contrast to normal dendritic cells. The lack of ability of LCH Langerhans cells in stimulating T cells is even more surprising in light of their expression of a variety of costimulatory molecules. If Langerhans cells in LCH lesions are not responsible for T cell activation and cytokine expression, then T cell activation may be the result of either a nonconventional antigen stimulation or secondary to nonantigenic, activating signals secreted from LCH Langerhans cells.

The genetic clonality of Langerhans cells in LCH lesions is now well established. Although the results from Dr. Chu demonstrating the clonal origin of dendritic cells from large patches of normal skin remain intriguing, they do not appear to alone explain why Langerhans cell from all LCH lesions thus far examined and polymorphic for the HUMARA locus have been clonal. Thus, the significance of clonality in LCH remains uncertain. Perhaps future molecular experiments will need to focus more on the possibility of demonstrating somatic genetic mutations in the Langerhans cells in LCH.

Although much of the symposium was devoted to the analysis of apoptosis, there was consensus that much more basic information was needed in our understanding of mechanisms regulating programmed cell death before its relevance to LCH could be postulated. However, despite the various methods to measure apoptotic cells, morphology remains perhaps the most definitive one. In addition, mitotic rate should also be measured in the same sections in which apoptotic cells are enumerated. Significant numbers of patients will be needed to make correlations of levels of apoptotic or proliferating cells and therapeutic responses.

The need for an animal model of LCH remains. Injection of cells from LCH lesions into immunodeficient mice has thus far not provided a means by which to propagate the LCH Langerhans cell *in vivo* or a definitive rondel for the human disease. Thus far, injecting cells from LCH lesions into immunodeficient mice with or without dendritic cell stimulating cytokines has not been successful in this regard. Another approach might be to focus work on generating animal models through homologous knockout methods or generating transgenic mice over-expressing other genes. In this respect, the Rel B work presented at this meeting may be a good starting point. Unless a flash of serendipity occurs, however, this approach will take a great deal of basic investigative work on which genes are expressed and critical for different stages of dendritic cell development. Other approaches being examined are to immortalize dendritic cells, Langerhans cells and

LCH Langerhans cells using retroviral approaches. These studies should contribute greatly to our ability to better understand both gene expression and possible genetic changes which occur in LCH.

Another area of great importance is the identification of better and hopefully biologically based prognostic markers. Critical to the success of this goal will be advances in the basic biology of dendritic cells as well as the ability to obtain sufficient tissue from LCH lesions and normal cells for study. Tied into this is the somewhat controversial issue of whether to do repeat biopsies or obtain tissues from multiple sites at a time when the information obtained is likely not to have any direct benefit to the patient. Therefore, such biological studies should be preferably incorporated into clinical trials and discussed early in their development with institutional IRB committees. The need for a tissue repository and directory remains a unsolved problem. Suggestions were made to expand the tissue bank through the Cooperative Human Tissue Network in Columbus, Ohio, as well as to create a separate bank for just LCH tissues. Another possibility would be to link LCH studies with other cooperative clinical trial groups to stimulate tissue procurement through a reward system. Linked to the need for usable tissue repositories is the additional need for national and international registries for patients with LCH. These registries should be computer based and data easily exchangeable but protected in terms of confidentiality and access. This type of registry should also be closely integrated with the physicians caring for the patients.

The meeting was a tremendous success in defining areas of controversy and the experiments needed to resolve them. In addition, there was a growing consensus in terms of issues concerning clonality, cytokine expression and most importantly, future areas for further investigation. Ending with more questions than one began with is probably the best mark of success.

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