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# Studies on the Mechanisms of Chemically Induced Leukemia/Lymphoma

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**Leukemias associated with potential exposure to chemicals or viruses are a major concern in modem society. The mechanism of leukemogenesis in rodents is poorly understood, making risk assessment of human exposure difficult. Studies at CIIT are attempting to elucidate the respective roles of chemical exposure and retroviruses in leukemogenesis in order to evaluate the appropriateness of animal models for extrapolating risk to man and to provide information necessary to identify human populations at risk.**

## The Author

Dr. Irons joined the staff of CIIT in January of 1977 and is currently a senior scientist in the Department of Cellular and Molecular Toxicology. He is a recognized authority on blood and bone marrow toxicity and is author of numerous scientific articles and book chapters on the subject. He holds membership in several scientific and professional organizations, is past chairman of the NIH Toxicology Study Section, and is a member of the EPA Health Effects Review Panel and the Toxicology Information Program Committee of the National Academy of Sciences.

that normally resides in the bone marrow. This "pluripotential stem cell" (PSC) is capable of either replicating itself or giving rise to any of the various cell types found in the peripheral blood and immune system. To appreciate this feat. one should consider that in the normal adult the frequency of PSC in the bone marrow is between 1 in 1000 and 1 in 10,000 cells, yet the bone marrow replaces over 10,000,000,000 ($10^{11}$) blood cells every day and is capable of increasing this Output as much as sixfold in order to meet increasing demands. The Process through which this occurs involves a complex interaction of growth factors, accessory cells and feedback mechanisms that regulate the differentiation and replication of blood precursor cells and in turn the production of mature blood cells (Fig. 1).

or viruses—invariably involve the hematopoietic (blood producing) stem cells that reside in the bone marrow and ultimately give rise to the cells of the blood and immune system. It is now recognized clinically that acute leukemia is often preceded months or even years by functional abnormalities of the blood and bone marrow, and that bone marrow suppression, aplastic anemia and myeloid leukemia represent a continuum or spectrum of blood dyscrasias (abnormalities) rather than separate or unrelated entities. Since these functional changes involve abnormalities in the growth of blood precursor cells, they have been collectively termed *myelodysplastic syndrome* (MDS) (Galton. 1986).

MDS describes a variable but well-

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recognized set of blood disorders that have high propensity for terminating in acute myeloid leukemia (AML). Previously defined diseases that are included within MDS are aplastic or refractory anemia and preleukemia (Galton. 1986). Frequent signs include: anemia, leukopenia (a decrease in white blood cells), and thrombocytopenia (decrease in platelets), accompanied by increased cellularity of the bone marrow. The rate of transformation of MDS to AML varies from study to study but is about 30% in "spontaneous" MDS and between 50 and 70% in MDS occurring secondary to chemotherapy or benzene exposure. The relationship between MDS and benzene leukemogenesis appears to be exceptionally strong, with MDS preceding the onset of leukemia in virtually every case for which data is available (Bagby, 1986). However, determining whether MDS precedes every case of benzene-induced leukemia remains an impossibility. It should be pointed out that secondary MDS itself is considered a neoplastic process and carries a grave prognosis, with a mortality of between 30 and 40% for those cases that do not progress on to AML.

Evidence exists to indicate the great majority of cases of MDS and most leukemias are the result of abnormalities originating within a single stem cell, *i.e.*, they are clonal in origin (McCulloch *et al.*, 1982). Studies utilizing cytogenetic or biochemical markers to identify the origins of abnormal cell populations have revealed that such clonal abnormalities may be present for years prior to the clinical recognition of leukemia. Thus a paradigm has emerged that implicates the stem cell in a multistep process that ultimately leads to the presentation of leukemia. Elucidation of the molecular events that precede leukemogenesis is a goal yet to be achieved. However, it seems reasonable to assume that at least one fundamental lesion must involve structural changes at the level of the genome (*i.e.*, a mutation) of the stem cell. Certainly, the Propensity of alkylating agents to cause leukemia, the prevalence of chromosomal abnormalities in leukemias and their clonal origin provide a rational basis for this concept. However, since many leukemias are characterized by what appear to be normal blood cells that are abnormally regulated with respect to growth and maturation, it has been Postulated that the genetic information may be normal but regulation defective. In this regard it is probably not insignificant that certain normal cells of the hematopoietic and immune systems (*e.g.*, lymphocytes) have characteristics usually ascribed to cancer cells, such as the ability to replicate, invade other

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Schematic Diagram of Hematopoiesis

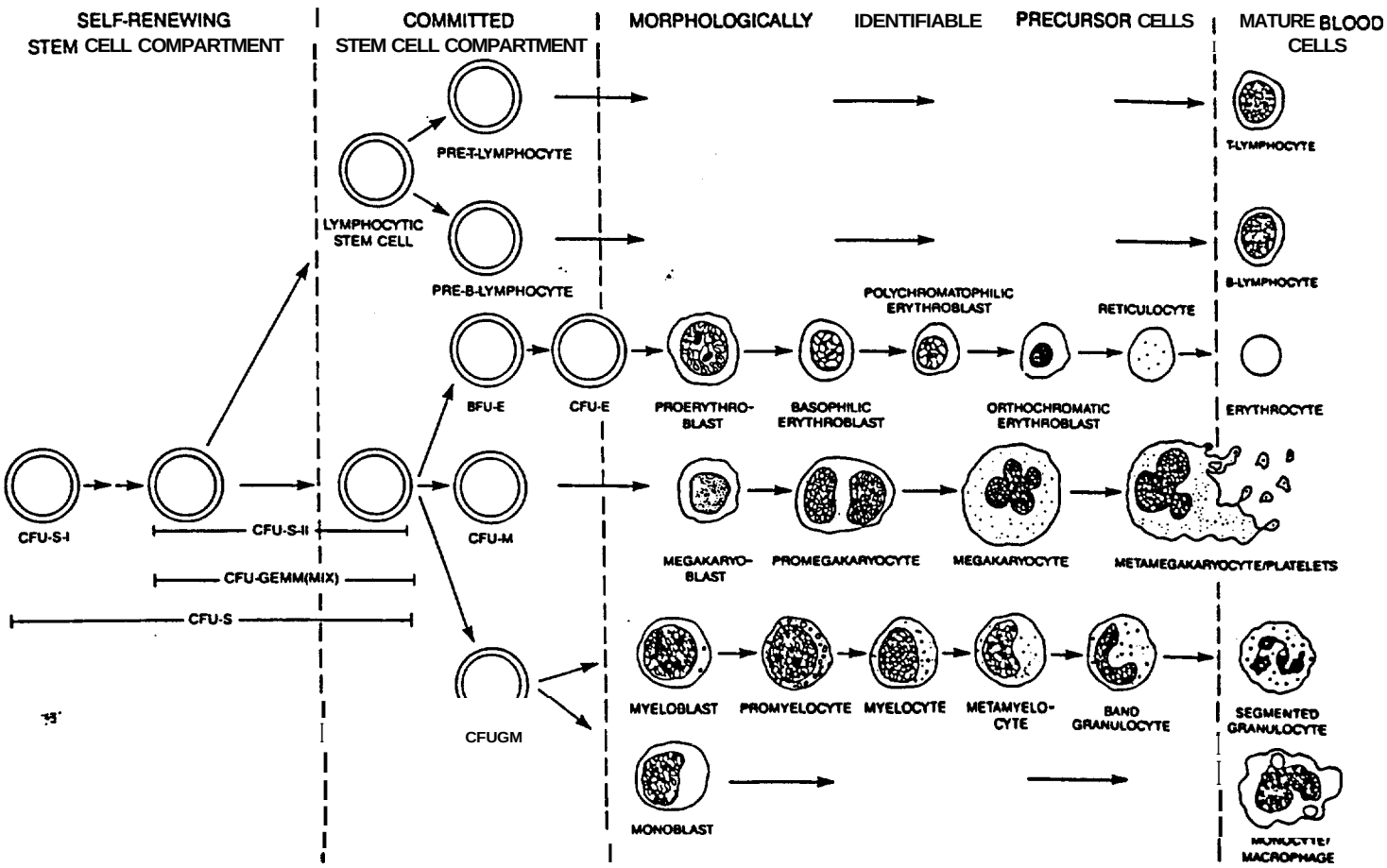


Fig. 1 The production of blood cells involves the progressive maturation and replication of precursor cells in the bone marrow. This process begins with the pluripotential stem cell (PSC), which has limited replicative ability but is capable of self-renewal. Alternatively, it can differentiate to produce cells committed to any of the different blood cell pathways. Differentiation along a committed pathway is accompanied by a progressive increase in replication such that the initial division of a PSC is amplified a billion-fold prior to the appearance of mature blood cells.

Various assays have been described that measure stem cells by determining their ability to form colonies (*i.e.*, colony-forming units—CFU). Several of these have been found to measure stem cells at overlapping stages in the commitment process. Assays that measure stem cells with the capability of self-renewal include CFU-S (spleen) and CFU-mix (or granulocyte, erythrocyte, megakaryocyte, macrophage). Assays that measure multipotential and committed stem-cell populations include: BFU-E (burst forming unit-erythrocyte), CFU-E (erythrocyte), CFU-M (megakaryocyte), and CFU-GM (granulocyte-macrophage).

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tissues, kill cells, etc., but that the number and activity of these cells are normally held in check by highly effective and redundant control mechanisms.

Since clonal abnormalities can be present for long periods of time prior to the onset of frank leukemia. there is considerable likelihood that both hypotheses are correct. It may be that structural damage to the DNA results in a heritable defect that conveys a proliferative advantage on a target stem cell, presumably via altered regulation of any number of potential cellular "oncogenes," but ultimate expression of the malignant phenotype requires intervening influences that favor or select for abnormal gene expression but are not necessarily themselves genetic events.

## Viruses and Leukemia

In John Steinbeck's tragedy *Of* Mice and Men, the mouse stands as a symbol of man's inability to understand and control his destiny. To almost everyone involved in the study of leukemogenesis, the analogy is an undeniably apt one. Murine models of leukemogenesis present perhaps the best opportunity to unlock the, mysteries of this disease. Nevertheless, the investigator attempting to use these animal models is confronted with a puzzle of Gordian proportion. One of the culprits implicated in this dilemma is a family of retroviruses, commonly referred to as murine leukemia viruses (MuLV). Retroviruses are RNA viruses that commandeer the normal machinery of a cell to produce a complementary piece of DNA which is then inserted into the nuclear DNA of the cell. Once this proviral DNA sequence is integrated into the cell, it becomes a permanent part of its genome, encoding for the production of retrovirus (Fig. 2). In a great number of species, including man, retroviruses have been implicated in many diseases of the blood and immune system, including leukemia and acquired immune deficiency syndrome (AIDS) (Table 1). The first human retrovirus, T-cell lymphotrophic virus (HTLV-I) was discovered in 1978 (Poiesz et *al.*, 1980; Hinuma et *al.*, 1981; Popovic *et al.*, 1982). It causes adult T-cell leukemia and to date appears to be restricted to southeastern Japan, the Caribbean, certain regions in Italy. the southern United States, South America and Africa.

HTLV-I is an infectious retrovirus that is passed horizontally, *i.e.*, from one person to another. As such it is an exogenous retrovirus, capable of being transmitted from one blood cell to another, but is not transmitted vertically from one generation to another through the germ cells. If the target of a retrovirus happens to be an ovarian or testicular germ cell, the encoded proviral sequence will be inherited as a "normal" gene by all further progeny. Retroviruses inherited in such a manner are called "endogenous" to distinguish them from exogenous or infectious retroviruses. None of the known infectious human retroviruses (*e.g.*, HTLV-I, -II, HIV) are known to target germ cells and thus to be transmitted vertically. Nevertheless. virtually all mammals, including man, possess some DNA sequences encoding for retroviral elements. In some species, including man, endogenous sequences are rare and there is yet no evidence to indicate that they are expressed. However, it is a legacy of the great majority of strains of laboratory mouse to possess numerous genes encoding for retroviruses. This includes the CD1, C57BL/6 and C3H strains as well as the B6C3F1 hybrid frequently used in carcinogenicity studies. Certain of these endogenous retroviruses are known to produce a high frequency of "spontaneous" leukemias in specific strains of mice. *e.g.* AKR and C58 (Hartley *et al.*, 1977; Chattopadhyay et al., 1982). are implicated in certain strain-specific models of radiation-induced leukemogenesis (C57BL/6, C3H and RF) (Gross, 1959; Leiberman and Kaplan, 1959; Kaplan, 1967; Upton *et al.*, 1958; Haran-Ghera, 1976) and appear to influence the incidence of 1,3-butadiene-induced leukemia in the B6C3F1 mouse (Irons et *al.*, 1988). In spontaneous leukemias of the mouse, the role of retrovirus is definitive (Cloyd *et al.*, 1980), yet it must be said that the specific mechanisms or role played by virus in any of the other models of leukemogenesis remains obscure. And in at least one model, 3-methylcholanthrene-induced leukemias in the RF mouse, no convincing role for retrovirus has yet been established (Goodenow and Lilly, 1984: Chinsky e? *al.*, 1985). A characteristic common to all naturally occurring mammalian retroviruses, both exogenous (*e.g.*, HTLV-I) or endogenous (*e.g.*, MuLV), is that they do not contain a directly transforming gene or "oncogene" in their coding sequences. Therefore, any proposals to account for the role of these viruses in leukemogenesis must invoke alternative explanations.

**Infection** Of a Target Cell by a Retrovirus

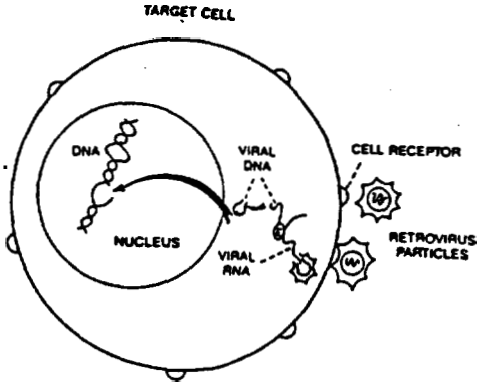


**Fig. 2. A retrovirus targets and enters cells by interacting** with receptors on the cell plasma membrane. Upon entering the Cell, the retrovirus releases its RNA Into the cytoplasm. A viral enzyme, reverse transcriptase, catalyzes the synthesis of DNA complementary to the viral RNA, which is then integrated into the cell's nuclear DNA. Thus the information encoded in the viral RNA is passed on to any daughter cells produced through subsequent division of the cell.

## Chemicals and Leukemogenesis

A number of chemicals and drugs are known to cause bone marrow toxicity in man or experimental animals, and it is therefore not surprising that they have been implicated in experimental leukemogenesis. These include benzene, butadiene. ethylene oxide. and a wide variety of alkylating agents. To-date, benzene is the only occupational hazard definitel linked to acute leukemia in man. Although seemingly unrelated, all of these agents share one or more characteristics in common: They all either distribute to the bone marrow in appreciable concentrations or give rise to reactive metabolites with relatively long half-lives in biological systems. These metabolic intermediates, in turn, are able to survive transit to the bone marrow and are capable of further bioactivation or direct interaction with cells in the target tissue. From the perspective of

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**TABLE 1**

RETROVIRUSES CAUSING **PATHOLOGY IN NATURAL HOSTS**

| Virus | Diseases |
|---|---|
| 1. Feline Leukemia Virus (FeLV) | • Leukemia, anemia, immunosuppression |
| 2. Bovine Leukemia Virus (BLV) | • Lymphoma |
| 3. Mouse Leukemia Virus (MuLV) | • Leukemia. lymphoma |
| 4. Simian Lymphoma Virus (SLV) | • Lymphoma |
| 5. Human T-Cell Leukemia Virus I (HTLV-I) | • Adult T-cell leukemia |
| 6. Human T-cell Leukemia Virus II (HTLV-II) | • Histiocytic/reticulum cell leukemia |
| 7. Equine Infectious Anemia Virus (EIAV) | • Anemia |
| 8. Visna Virus | • Neurological and pulmonary disease m sheep |
| 9. Human Immunodeficiency Virus (LAV/HTLV-III/HIV) | • AIDS |

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factors, including retrovirus background. the regimen of chemical exposure, and its duration may be important considerations in carcinogenesis that have so far received little attention. Understanding the relationship between chemical toxicity, retroviral genes and leukemogenesis will aid in providing an informed evaluation of both the appropriateness of the mouse as a model for extrapolating risk of leukemogenesis to man and the relative importance of chronic high versus low level or transient chemical exposures with respect to human health. Such information is critically important for the development of sound regulatory and industrial exposure policies.

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all practical purposes, be reduced to two: either activated retroviruses and BD are independently leukemogenic, or MuLV is co-carcinogenic, *i.e.*, a significant influence but not by itself leukemogenic in this chemical leukemogenesis model (Table 2). These alternatives can be tested experimentally. In addition. it becomes necessary to determine how valid are mouse models of retrovirus activation as predictors of the potential of chemicals to alter the biology Of retroviruses, such as HTLV-I or HIV, in human cells. Although little is known about the mechanism of HTLV-I transformation, evidence strongly suggests that environmental factors influence latency in HTLV-I-associated neoplasms, and chemically induced alterations in HTLV-I antigen expression has been reported in human cells *in vitro*. By examining the potential of butadiene or its metabolites to alter the biology of HTLV-I in human blood cells in culture. we hope to clarify the significance of the mouse retrovirus model to man and epidemiologic findings that may suggest a population at potential risk. In addition to qualitative analyses of the effects of chemicals on viruses in human and mouse cells, it may be possible to quantitate relative differences in species sensitivity at a cellular level. This would be of considerable value in extrapolating *in* vivo animal data to man.

## Benzene

Since late in the last century, occupational exposure to benzene has been associated with bone marrow toxicity (Santesson, 1897). The most frequently reported effects have been cytopenias (a decrease in one or more of the different types of circulating blood leukocytes or white cells), anemia and pancytopenia or aplastic anemia (a severe and usually irreversible bone marrow suppression culminating in the inability to effectively replace any of the cellular elements of the blood) (Goldwater. 1941). The first report of leukemia associated with benzene 'exposure appeared in 1928 (Delore and Borgomano, 1928). yet benzene was not universally recognized as a human leukemogen until the mid-70's. Recognition appears to have awaited two independent developments: (1) Although scattered individual cases appeared in the literature, it remained for Aksoy et *al.* and Vigliani and Saita to provide the first organized studies of benzene exposure in the workplace: and (2) this has coincided with relatively recent recognition that MDS and AML represent a continuum or spectrum of blood dyscrasias rather than separate or unrelated entities.

The principal neoplastic disease now associated with benzene exposure is acute myelogenous leukemia (AML) (Vigliani and Forni, 1975; Infante et *al.*, 1977). Benzene-induced AML's are predominantly myeloblastic (FAB classification-M1), although a Particularly strong association has been established between benzene exposure and the erythroleukemic variant of AML (FAB classification-M6) (Galavotti and Roisi, 1950; Aksoy et *al.*, 1976). Further studies have suggested an association with lymphoma as well (Aksoy *et al.*, 1974; Vianna and Polan. 1979; Bernard, 1942). From the results of these and other studies one can safely conclude that occupational exposure to benzene is associated with an increased risk of hematoloaic neoplasms,

Despite the weight of evidence implicating benzene as a leukemogen in man, there is no clear understanding of the pathogenesis of the disease or the quantitative relationship between benzene exposure and leukemogenesis. Although chronic exposure to 100 ppm benzene is known to result in bone marrow damage and an increased incidence of leukemia in man, the significance of exposure to low concentrations of benzene is controversial. A prevailing sentiment among clinical hematologists is that blood dyscrasias indicative of bone marrow damage frequently, if not invariably, precede the development of secondary AML. However, it presently cannot be concluded that frank bone marrow damage is an absolute prerequisite in every case. Thus, the evidence linking blood dyscrasias or leukemia with exposure to benzene at lower concentrations in man is controversial, and there is little agreement among clinicians, epidemiologists. and researchers as to the significance of exposure to benzene at low concentrations (Infante et *al.*, 1977; Harris, 1977). It is not known, for example, whether bone marrow suppression, frequently seen at higher exposure concentrations, is a requirement for the evolution of AML, implying a threshold below which benzene is not leukemogenic, or alternatively, whether there is no effective threshold for benzene. This debate is not likely to be resolved without an increased understanding of the mechanisms of leukemogenesis.

Historically, such studies have been hampered by the lack of an appropriate animal model for benzene-induced leukemogenesis. To varying degrees, investigators have been successful in demonstrating temporary or reversible bone marrow toxicity following benzene exposure in rats and mice (Snyder et *al.*, 1978: Snyder et *al.*, 1982a.b: Goldstein et *al.*, 1982; Cronkite *et al.*, 1984; Cronkiteet *al.*, 1985; Green et *al.*, 1981). Nevertheless, previous attempts to produce a useful leukemia model have been disappointing. However, as a consequence of recent studies conducted by Dr. Eugene Cronkite at Brookhaven National Laboratories and results obtained in experiments at CIIT, it would now appear that these difficulties have been largely overcome.

Early studies at CIIT revealed that toxicity of benzene or its metabolites to bone marrow cells *followed a pattern exhibited by certain cancer chemotherapeutic agents that are toxic to cells only at certain stages in their growth cycle (Ironset *al.*, 1979). The proportion of cells affected following exposure to such a cycle-specific agent is governed by the number of cells in a sensitive phase, regardless of the dose of the compound administered (Ironsand Horan, 1978). For such agents, the regimen or temporal pattern of exposure may be equally as significant as the actual exposure.. concentration.

While investigating the effects of concentration and duration of exposure on benzene-induced bone marrow suppression in mice, Dr. Cronkite and his co-workers adopted an exposure regimen that deviated significantly from the traditional lifetime exposure protocols previously used to study the leukemogenicity of benzene. They exposed mice for 16 weeks to 300 ppm benzene and held them for lifetime observation. TWO models Of benzene-induced leukemia have been described by Cronkite using this regimen: thymic lymphoma/leukemia in the C57BL/6 mouse, a mouse known to carry MuLV and found to have a high incidence of this tumor type following radiation exposure: and myelogenous leukemia in the CBA/Ca mouse, which has a very tow spontaneous incidence of AML but is known to develop a high incidence of AML after exposure to radiation (Cronkite, 1987).

Most recently, in experiments again intended to characterize the regimen-dose dependence of benzene bone marrow toxicity, we have obtained preliminary results that confirm and extend the use of the C57BL/6 mouse as a model for benzene leukemia. In these experiments, mice were exposed to 100 or 300 ppm, either 3 or 6 days/week for 12 weeks and held for observation. At approximately 6 months, MDS was encountered in animals in each of the exposure groups. Included m the spectrum of hematopoietic lesions encountered were not only T-cell lymphoma/leukemia but myelogenous leukemia as well (Cathro *et al.*, 1988). With these models we are now afforded an opportunity to investigate systematically potential mechanisms of benzene toxicity to the bone marrow and leukemogenesis.

The history of research in chemical leukemogenesis suggests that numerous

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factors, including retrovirus background. the regimen of chemical exposure, and its duration may be important considerations in carcinogenesis that have so far received little attention. Understanding the relationship between chemical toxicity, retroviral genes and leukemogenesis will aid in providing an informed evaluation of both the appropriateness of the mouse as a model for extrapolating risk of leukemogenesis to man and the relative importance of chronic high versus low level or transient chemical exposures with respect to human health. Such information is critically important for the development of sound regulatory and industrial exposure policies.

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# Service Awards Presented

**Twenty-six CIIT employees were recognized for five and ten years' service at the Institute's Christmas party on December 12.**

Reaching the ten year mark were (seated, left to right): Dr. Richard J. Levine, Epidemiology; Charles A. Howard, Building Services; Dr. Henry d'A. Heck, Biochemical Toxicology and Pathobiology; Dr. Byron E. Butterworth, Genetic Toxicology; Betsy Gross Bermudez, Experimental Pathology and Toxicology; and Diane J. Abernethy, Genetic Toxicology.

Standing, left to right: Edna A. Mangum, Administration; Delorise H. Williams, Experimental Pathology and Toxicology; Corrie N. Smith, Experimental Pathology and Toxicology; Charles M. Overton, Purchasing; and Dr. Douglas E. Rickert, Biochemical Toxicology and Pathobiology. Not pictured is Donald F. Deyo, Biochemical Toxicology and Pathobiology.

**Recognized for five years' service were (seated, left to right): Gary Trent Jones, Experimental Pathology and Toxicology; Betty A. Honeycutt, Genetic Toxicology; Linda K. Garvey, Experimental Pathology and Toxicology; Karen M. Dold, Cellular and Molecular Toxicology; Dr. Jack H. Dean, Cellular and Molecular Toxicology; and Kaye S. Nagy, Purchasing.**

**Standing: R. Dwight McFarland, Building Services; Carl U. Parkinson, Jr., Experimental Pathology and Toxicology; Dr. Thomas R. Skopek, Genetic Toxicology; Dr. Frank Welsch, Cellular and Molecular Toxicology; and Donald B. Stedman, Cellular and Molecular Toxicology. Five-year employees not pictured are Dr. William F. Greenlee, Cellular and Molecular Toxicology; Lloyd D. Lauer, Cellular** and Molecular Toxicology; and Mary S. Morris, Experimental Pathology and Toxicology.