Journal of Cellular Biochemistry 18:15-24 (1982)
Cellular Recognition 479-488

Growth of Human T Lymphocyte Colonies From Whole Blood: Culture Requirements and Applications

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Growth of human lymphocyte colonies from whole blood following stimulation with PHA, Con A, or PPD is described. Individual colony cells were identified as T lymphocytes on the basis of surface marker and enzyme cytochemical characterizations. Colony formation increased as a power function over a wide range of cell concentrations above a critical minimal concentration. The whole blood culture system eliminates possible selective effects of lymphocyte colony techniques utilizing gradient-enriched lymphocyte fractions and more closely approximates the in vivo milieu. The whole blood colony method is more sensitive for the detection of low-level radiation effects on lymphocytes than widely used tests that measure ^{3}H-thymidine incorporation. In preliminary studies, we used the whole blood method to determine the relative radiosensitivity of lymphocytes from humans with various hematopoietic disorders, and observed abnormalties in mitogen responsiveness and colony formation in some of the patient groups. This method has wide application for studies in cellular and clinical immunology.

Key words: T lymphocytes, colonies, in vitro, human, whole blood

Lymphocyte-colony-formation assays provide a powerful new technology for the analysis of functional subpopulations of T lymphocytes, characterization of the T cell receptor repertoire, and study of the induction and amplification of T cell function. Indeed, the ability to grow clones of T cells may be potentially as valuable to cellular immunology as monoclonal antibodies are to studies of humoral immunity. Furthermore, T lymphocyte cloning techniques may be utilized to assess the immunological status of patients with various immunological disorders. Such an approach is warranted in view of recent studies demonstrating that colony methods are more sensitive for measuring T lymphocyte proliferation than are standard methods that measure incorporation of radiolabeled DNA precursors [1-3].

Received April 28, 1981; accepted August 21, 1981.

0730-2312/82/1801-0015$03.00

In vitro assays should accurately reflect the in vivo status of relevant cellular populations for meaningful study and characterization of T cell progenitor cells. Gradient-enriched lymphocyte fractions, however, are routinely used for the growth of T lymphocyte colonies [4,7]. Cellular separation methods eliminate erythrocytes and granulocytes, and may nonrandomly select and/or delete specific subpopulations of lymphocytes and monocytes. Therefore, the representation of cellular subpopulations in enriched lymphocyte fractions may be skewed and differ quantitatively and qualitatively from the in vivo milieu [8]. Selective removal of lymphocyte subpopulations and nonlymphoid accessory cells may also preclude cellular interactions and production of soluble mediators.

In an attempt to obviate these problems, we have developed methods for the growth of canine [2,9] and human [2,10] T lymphocyte colonies from whole blood (WB) and describe here some of the culture requirements and applications of the latter method. The growth of lymphocyte colonies from WB is more advantageous than methods using isolated lymphocytes since the WB cultures contain the full complement of circulating blood cells and provide a system for the study of T lymphocyte progenitor cells that more closely approximates the in vivo environment. Furthermore, this method is more sensitive than either colony formation using separated lymphocytes or tests that measure ^{3}H-thymidine (^{3}H-TdR) incorporation for the assessment of differential mitogen responsiveness and the detection of low-level radiation effects on lymphocytes [1,2].

MATERIALS AND METHODS

Culture of Whole Blood Colonies

Peripheral blood was collected from clinically normal adults and patients with Fanconi's anemia, CLL, myeloma, CML, AML, and beta-thalassemia. Blood was aseptically drawn into preservative-free sodium heparin (Calbiochem, San Diego, Calif; 25 USP units/ml) and diluted to a final concentration of 3% using RPMI-1640 (Grand Island Biologic Co, Grand Island, NY) containing 1% antibiotic-antimycotic (GIBCO), 1% L-glutamine (GIBCO), 10% autologous plasma, and 0.3% Sea-plaque agarose (Marine Colloids, Rockland, Me). Methylcellulose (1.2%; Dow Chemical Co, Midland, Mich) was substituted for agarose for rosette characterization experiments.

Lectins were added to the blood to give optimal final concentrations of 80 μg/ml PHA-P (Difco Laboratories, Detroit, Mich), 125 μg/ml Con A (Calbiochem), or 50 μg/ml PPD (Connaught Laboratories, Swiftwater, Pa). Cell suspensions were plated in 35 × 10-mm culture dishes (Corning Plastics, Corning, NY). Following incubation for seven days at 37°C in 5% CO_2 humidified air atmosphere, cultures were stained with new methylene blue (0.25%) and colonies containing more than 40 cells were counted with a 40× dissection scope. Methods for the cloning of gradient-separated mononuclear cells have been previously described [7].

Lymphocyte Stimulation Test

The WB canine lymphocyte stimulation test (LST) [11] was modified for human cells. Briefly, 50 μl of blood was added to 0.95 ml containing 25 μg/ml PHA-P (Difco Lab), 25 μg/ml Con A (Calbiochem), or 15 μg/ml PPD (Connaught

Laboratories). Cell suspensions were pipetted in 0.2-ml aliquots into wells of sterile microtiter plates (Microbiological Assoc, Bethesda Md). On the fifth day of incubation, cultures were labeled with 1 μCi ^{3}H-thymidine (New England Nuclear, Boston, Mass) per well in a 10 μl volume. Eighteen to 20 hours later, cells were harvested on a six-well Skatron harvester (Flow Laboratory, Rockville, Md). The samples were placed in toluene-based scintillation fluid, and the radioactivity measured in a β-scintillation counter.

Characterization of Colony Cells

The E- (erythrocyte) and EAC (erythrocyte-antibody-complement) rosette tests were used to identify T and B lymphocytes. Standard rosetting techniques were used to enumerate E- and EAC rosette-forming cells [12]. Colony cells were washed from methylcellulose cultures with suspension MEM (GIBCO) and collected by centrifugation. RBC were lysed with 0.83% NH_4Cl, and the resulting leukocyte suspensions were used for rosetting assays.

Monocytes were identified by their ability to phagocytize 1 μm latex beads (Dow Chemical Co, Indianapolis, Ind). Methods for the in situ cytological characterization of colony cells using Wright's-Leishman stain, determination of specific and nonspecific esterase activity, and ultrastructural examination using transmission electron microscopy have been previously described [9].

X-irradiation

In some experiments, WB was X-irradiated with exposures of 0–400 R (73 R/min by a Maximar 250-III (GE, Milwaukee, Wis) unit. Irradiation was done at 250 KVP, 15 mA using a Thoraeus II filter (GE).

RESULTS

The highest numbers of colonies were observed at days 7–8 as previously reported [2,10]. Such colonies were composed entirely of lymphocytes, as shown by the electron micrograph in Figure 1, and contained both lymphoblasts and mature lymphocytes. RBC were occasionally trapped around the periphery of colonies.

Individual colony cells were identified as T lymphocytes on the basis of surface marker, cytological, and enzyme histochemical characterization [2,10]. WB colony cells formed an average of 78% E- but no EAC rosettes. Cells were negative for specific esterase activity, but most cells possessed granules exhibiting α-naphthylacetate esterase (ANAE) activity. Colony cells were also unable to phagocytize latex beads.

The results of three cell titration experiments, summarized in Table I, compare the initial cell concentration in the WB (absolute lymphocyte number) to subsequent colony formation. Colony formation followed a power function of the number of cells plated [2,10]. When the logarithm of the lymphocyte concentration (X) was plotted against the logarithm of the colony response (Y), a linear regression line was fit to the linear portion of the curve. The equation of this line was $Y = 1.43 \times 10^{-8}X^{2.46[\pm 0.15]}$ with a correlation coefficient of 0.98 [10]. Colonies were not observed below 5×10^3 lymphocytes plated in two of the three individuals studied. The highest numbers of colonies were observed when 5×10^4 to 1×10^5 lymphocytes were plated. The average number of lymphocytes contained in the 3% WB

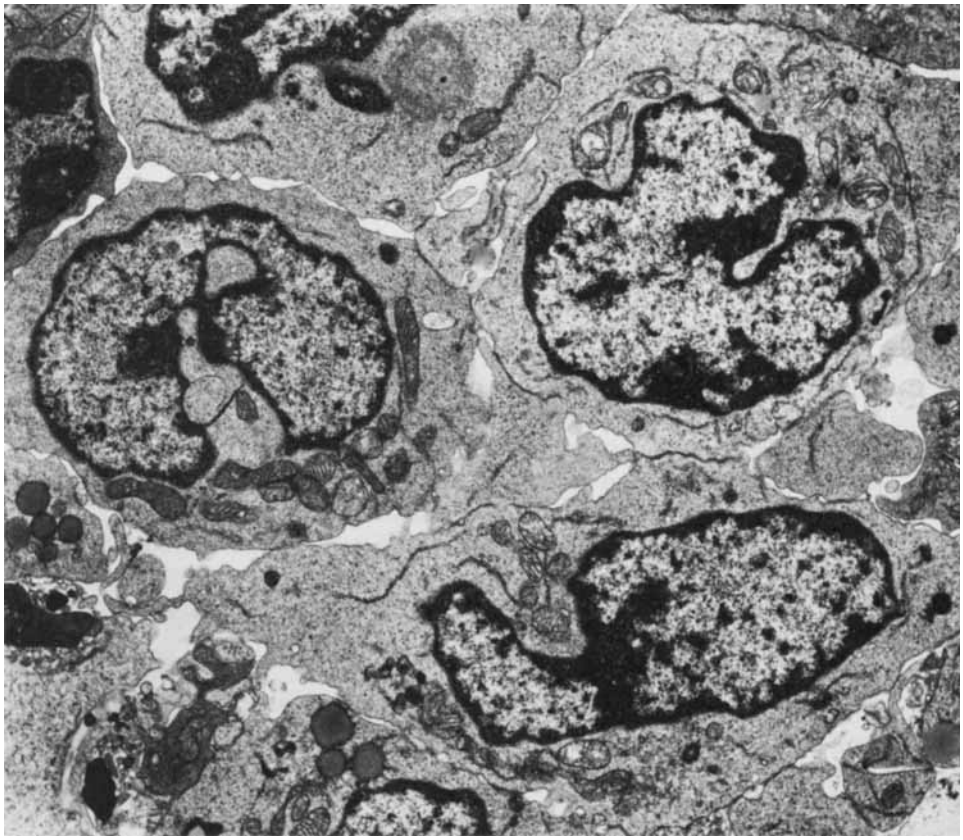

Fig. 1. Electron micrograph of lymphocytes in a large colony after seven days incubation (negative magnification ×5334).

that was routinely plated fell well within this range. A plateau in colony formation occurred above 10^5 lympohcytes plated [2,10].

Colony formation was calculated as a function of the absolute number of lymphocytes plated in 3% WB. High cloning efficiencies were consistently obtained when optimal concentrations of WB and mitogen were used [2,10]. Table II shows a marked increase in cloning efficiency using WB cultures compared to cultures of Ficoll-paque (FP) isolated lymphocytes. The results of preliminary studies comparing the cloning efficiencies of groups of normal individuals to patients with a variety of hematological diseases are shown in Table III. Abnormaltities in mitogen responsiveness and colony formation were observed in some of these patients. In studies of two CLL patients and a patient with active myeloma, the cloning efficiencies of lymphocytes were markedly decreased to levels of approximately 10%–40% of normal following stimulation with either Con A or PHA. Con A-stimulated colony formation by AML lymphocytes occurred at approximately 40% of the normal level, although this reduction was not statistically significant. It is noteworthy that most of the "colonies" from the CLL patients were in fact clusters, containing only 10–20 cells. Colony formation following Con A stimulation was increased by a factor of 4–5 in a CML patient and a patient with myeloma in remission. The cloning

TABLE I. Relationship of Number of Lymphocytes Plated to Number of Colonies Formed*

Absolute lymphocytes/ml[a]	Number of colonies >40 cells[b]
1×10^3	4 ± 4
5×10^3	23 ± 5
1×10^4	61 ± 16
2×10^4	773 ± 217
4×10^4	3,441 ± 305
5×10^4	4,387 ± 967
1×10^5	4,742 ± 1,375
2×10^5	3,087 ± 1,682

*Data from Knox et al [10].
[a]Cultures were stimulated with PHA.
[b]Values are expressed as $\bar{X}$ ± SEM and represent the means for blood samples from three individuals, each plated in triplicate or quadruplicate.

TABLE II. Relative Cloning Efficiency of Lymphocytes From Whole Blood (WB) and Ficoll-paque (FP) Gradient-Enriched Lymphocytes*

Mitogen/antigen	Cloning efficiency[a] FP / Cloning efficiency WB
PHA	2.4
Con A	4.5
PPD	39.2

*Data from Knox et al [10].
[a]Cloning efficiency = number of plated lymphocytes containing one progenitor cell (CFU-L) capable of forming one colony.

efficiencies of the other patient groups were either normal or moderately elevated (up to approximately two times the normal value). This elevation however was not significant.

Table IV compares the D_{37}* values of radiation survival curves from normal individuals with patients with hematological diseases using both the WB LST and WB colony formation assay. For all subjects and test groups studied (normal and patient), colony formation was considerably more sensitive than the LST for detecting radiation effects on lymphocytes. For example, the D_{37} for PHA-stimulated lymphocytes from normal individuals was 109 R in the WB colony assay in contrast to 309 R in the LST. Similarly, a D_{37} of 110 R was obtained for Con A-stimulated colony formation as compared with 267 R for the Con A LST. The increased radiosensitivity of the WB colony assay is further demonstrated by the lower D_{37} values and steeper slopes of the linear regression lines for radiation survival of lymphocytes in the WB colony assay as compared with the LST, for both normal subjects and patients with Fanconi's anemia (Fig. 2) [1].

* The D_{37} is the dose required to reduce the number of colony forming or proliferating cells by a factor of 1/e or 0.37.

TABLE III. T Lymphocyte Colony Formation in Whole Blood Cultures From Normal Subjects and Patients With Immunohematological Diseases

Human subjects (X ± SEM)	Con A		PHA	
	Cloning efficiency[a]	Control value^{-1}	Cloning efficiency[a]	Control value^{-1}
Normal values[b] (N = 15)	440 ± 140	1.00	110 ± 20	1.00
Patients				
Fanconi's anemia[b] (N = 4)	320 ± 130 P = 0.39[c]	1.38	50 ± 20 P = 0.08	2.20
CLL[d] (N = 2)	6,570	0.07	1,170	0.09
Myeloma				
active (N = 1)	3,360	0.13	300	0.37
remission (N = 1)	110	4.00	60	1.83
CML (N = 1)	90	4.88	70	1.57
AML (N = 3)	1,080 ± 560 P = 0.20	0.41	70 ± 20 P = 0.45	1.57
Beta-thalassemia (N = 2)	250	1.76	70	1.57

[a]Cloning efficiency = number of plated lymphocytes containing one progenitor cell (CFU-L) capable of forming one colony.
[b]Data from Knox et al [1].
[c]P values were obtained for comparisons of patient values with normal values using the Mann-Whitney U-test.
[d]Those "colonies" counted were primarily clusters, containing approximately 10–20 cells.

The data presented in Table IV and the linear regression lines for radiation survival of lymphocytes from normal individuals and patients with Fanconi's anemia (Fig. 2), demonstrate that the PHA-responsive lymphocyte subpopulation from Fanconi's anemia patients is significantly more radiosensitive than that from normals [1]. Increased lymphocyte sensitivity to in vitro X-irradiation also was observed in PHA-stimulated T lymphocyte colonies and the PHA/Con A LST in a patient with active myeloma. Lymphocytes from a myeloma patient in remission, however, were found to be more radiosensitive in only the PHA LST. AML patient lymphocytes were significantly more radiosensitive than those from normals in the WB colony assay, while lymphocytes from patients with CLL and CML were more radioresistant in comparison with normals in several of the tests. The D_{37} values for beta-thalassemia patients fell within the normal range.

DISCUSSION

These studies demonstrate that human T lymphocyte colonies may be directly grown from WB following stimulation with PHA, Con A or PPD. This technique eliminates the requirement for FP density gradient-separated lymphocytes and provides a sensitive system for the study of T lymphocyte progenitors that more closely approximates the in vivo milieu. There was a marked increase in the cloning effi-

TABLE IV. D_{37} Values of In Vitro Lymphocyte Radiation Survival for Normal Subjects and Patients With Immunohematological Diseases Using ^{3}H-TdR Incorporation and T Lymphocyte Colony Techniques

	LST (^{3}H-TdR incorporation)		T lymphocyte colonies	
Human subjects (X ± SEM)	Con A	PHA	Con A	PHA
Normal values[a] (N=15)	267 ± 16	309 ± 29	110 ± 13	109 ± 10
Patients				
Fanconi's anemia[a] (N=4)	229 ± 43 P=0.12[b]	198 ± 38 P=0.06	107 ± 13 P=0.31	53 ± 22 P=0.02
CLL (N=2)	270	400	143	119
Myeloma				
active (N=1)	194	117	ND[c]	72
remission (N=1)	245	131	ND	117
CML (N=1)	375	335	133	173
AML (N=3)	265 ± 19 P=0.24	269 ± 9 P=0.28	73[d]	69 ± 15 P=0.03
Beta-thalassemia (N=3)	259 ± 23 P=0.19	296 ± 55 P=0.39	ND	124[e]

[a]Data from Knox et al [1].
[b]P values were obtained for comparisons of patient values with normal values using the Mann-Whitney U-test.
[c]ND = not done.
[d]Value for one beta-thalassemia patient. The other two patients failed to form any colonies following stimulation of the WB cultures with Con A.
[e]Value represents the mean D_{37} for two patients.

ciency using WB as compared to FP cells [2,10]. The presence of erythrocytes in the culture system may contribute to the enhanced growth of colonies from WB. Addition of RBC [9,13,14] or RBC membrane preparations [15] to lymphocyte cultures has been previously reported to potentiate T lymphocyte colony formation. It is possible that soluble mediators or membrane interactions may be responsible for this enhancement.

Colony formation increased as a power function of the number of cells plated over a wide range of cell concentrations. Generally, colonies were not observed below 5×10^3 lymphocytes plated [2,10]. The absence of colonies at lower concentrations of WB may be indicative of a requirement for minimal cellular interactions or soluble mediator production. Previous studies suggest that factors released in conditioned media by lectin-induced blastogenesis have profound enhancing effects on the proliferation of T lymphocytes [16]. The addition of T cell growth factor (TCGF) has been demonstrated to significantly enhance proliferation and colony formation induced by plant lectins [17] and specific soluble antigens [18]. It is possible that soluble mediators such as TCGF may contribute to the increased cloning efficiency and a sensitivity of the WB culture system. This will be the subject of future investigations. The requirement of cellular interactions [15] may be determined from the slope of the regression line using the mathematical analysis method

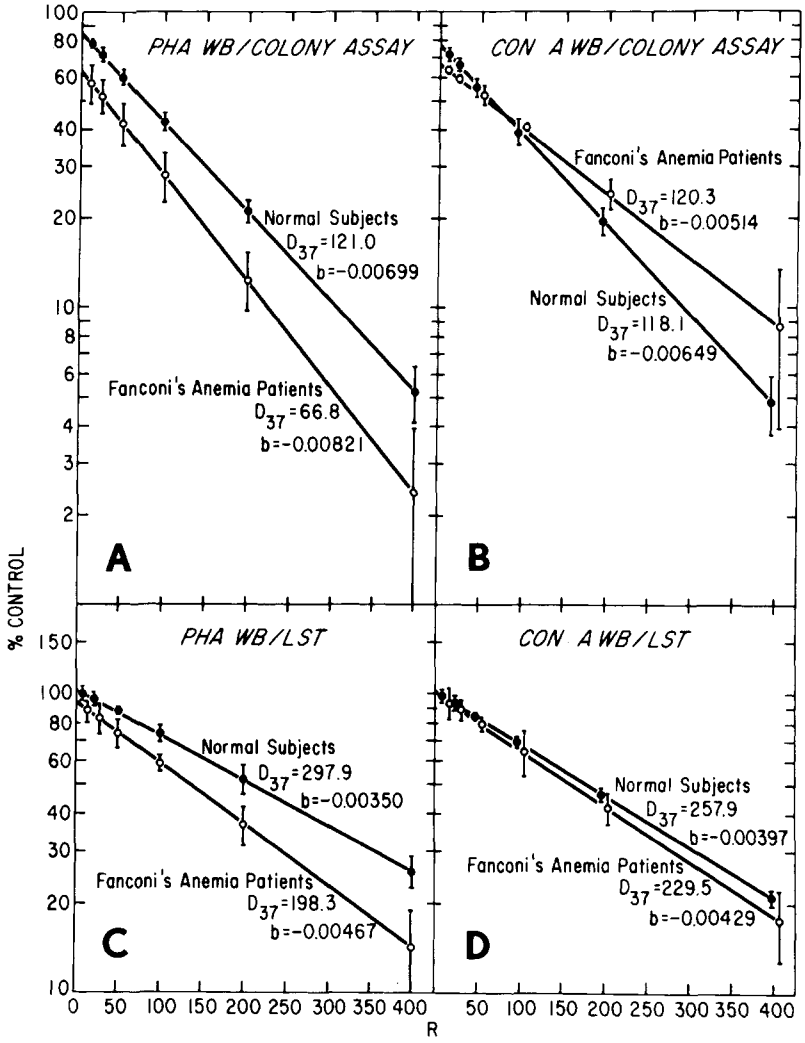

Fig. 2. Linear regression lines ($y = ne^{-bD}$) for the exponential portion of the radiation survival curves for lymphocytes from normal subjects and from patients with Fanconi's anemia, stimulated by A) PHA in the WB colony assay, B) Con A in the WB colony assay, C) PHA in the WB LST, D) Con A in the WB LST. The error bars represent X ± SEM. (Figure from Knox et al [1]).

of Coppleson and Michie [19]. The slope for the linear regression line for PHA-stimulated colony formation was 2.4, implying a requirement for interaction between at least two cells in the colony formation response [10].

These studies demonstrate that WB colony formation is a sensitive in vitro bioassay for the assessment of and the quantitation of radiation effects on the proliferative capacity of lymphocyte progenitor cells. The WB colony method is considerably more sensitive than either colony formation using gradient-isolated cells or techniques based upon ^{3}H-TdR incorporation for the measurement of in vitro radiation effects (lower D_{37} values) on lymphocytes [1,2,10]. Table IV and Figure 3 clear-

ly demonstrate the increased radiosensitivity of the WB colony assay as compared with the standard ^{3}H-TdR incorporation methods.

The WB colony formation assay was used to assess the relative radiosensitivity of lymphocytes from clinically normal individuals and groups of patients with hematopoietic disorders. In preliminary studies using this assay, abnormalities in WB colony formation also were observed in association with some of the disease states. For example, PHA-responsive lymphocytes from patients with Fanconi's anemia were found to be significantly (approximately twofold) more radiosensitive than lymphocytes from normal individuals, while PHA-stimulated colony formation was markedly increased [1]. The increased cloning efficiency (Table III) and radiosensitivity (Table IV) of PHA-responsive but not Con A-responsive lymphocytes from Fanconi's anemia patients suggest that this subpopulation may be functionally defective [1]. These findings are of particular relevance since both patients with Fanconi's anemia, and their relatives have an increased incidence of leukemia and solid tumors [20,21]. This method therefore may be useful for identification of individuals exhibiting increased sensitivity to chemical or physical mutagens.

A marked decrease in cloning efficiencies with Con A and PHA was observed in both CLL patients studied (Table III), while lymphocyte radiation survival was normal or increased. The presence of monoclonal malignant lymphocytes in the patients may account for these observations. The myeloma patients are of particular interest since dramatic differences were observed in both lymphocyte cloning efficiency and radiation survival between active and remission disease states. In contrast to some of the other disorders, increases in both cloning efficiency and in vitro radiation lymphocyte survival were observed in a patient with CML. It is noteworthy that lymphopoiesis in CML patients is apparently normal, although some patients develop a lymphoblastic transformation as the disease progresses. AML is another myeloid disorder in which lymphopoiesis is unaffected. In the three AML patients studied, however, Con A colony formation was depressed in comparison with normals, and patient lymphocytes were observed to be significantly more radiosensitive than normal lymphocytes in the T lymphocyte colony assay. Neither increased lymphocyte radiosensitivity nor decreased colony formation were observed in the beta-thalassemia patients. This was not surprising since abnormalities in beta-thalassemia are restricted to red blood cells and lymphocytes apparently are not involved in the pathology of the disease.

Preliminary studies of groups containing small numbers of patients do not permit critical evaluation and additional patients need to be studied for definitive disease-specific evaluation. Our observations, however, demonstrate the potential advantages of the new technique described here for the investigation of clinical disorders of lymphoid elements. The WB colony assay has wide application to studies of cellular and clinical immunology and may prove useful in the future as an additional prognostic/diagnositc indicator for certain disease states.

ACKNOWLEDGMENTS

This work was supported in part by the US Department of Energy and the US Nuclear Regulatory Commission. This study was approved by the Human Subject

Review Committee: Clinical and Physiological Research. The excellent technical and clerical assistance of Charles Baty, Shirley Coffelt, Roy Kroll, Lynne Morrin, Victor Pietrzak, and Ken Shiomoto is greatly appreciated. Figure 3 and part of the data in Tables III and IV were used by permission from Grune and Stratton, Inc (Knox SJ et al: Increased radiosensitivity of a subpopulation of T lymphocyte progenitors in Fanconi's anemia. Blood 57:1043, 1981).

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