Increased Radiosensitivity of a Subpopulation of T-Lymphocyte Progenitors From Patients With Fanconi’s Anemia

By S. J. Knox, F. D. Wilson, B. R. Greenberg, M. Shifrine, L. S. Rosenblatt, J. D. Reeves, and H. Misra

In vitro radiation survival of peripheral blood T lymphocytes was studied in 15 clinically normal adults and 4 patients with Fanconi’s anemia. Tritiated thymidine incorporation in a whole blood lymphocyte stimulation test (LST) and a newly developed whole blood T-lymphocyte colony assay were used to measure lymphocyte blastogenesis and colony formation in response to phytohemagglutinin (PHA) or concanavalin-A (Con-A) stimulation. Lymphocyte colony formation was found to be consistently more sensitive than the LST for detection of low-level radiation effects using both normal cells and lymphocytes from Fanconi’s anemia patients. Lymphocytes from patients with Fanconi’s anemia were significantly more sensitive to in vitro x-irradiation than lymphocytes from clinically normal individuals as measured by their ability to divide when stimulated by PHA in the LST (patients, $D_{37} = 198$ R; normals, $D_{37} = 309$ R, $p = 0.057$) and colony formation assay (patients, $D_{37} = 53$ R; normals, $D_{37} = 109$ R, $p = 0.016$). No significant difference in the radiosensitivity of the Con-A response was observed between the two groups. The PHA-responsive T-lymphocyte subpopulation in Fanconi’s anemia patients appears to be intrinsically defective. The nature of this defect, significance in the disease process, and relevancy of these findings to the establishment of radiation protection standards are discussed.

We have previously shown that hematologic and immunologic parameters may be used as sensitive indicators of radiation dose and dose-rate effects. We also have shown that the whole blood (WB) lymphocyte colony formation assay and the WB lymphocyte stimulation test (LST) are among the most sensitive methods available at present for the assessment of radiation effects on human lymphocytes. Therefore, these two methods were utilized to evaluate the quantitative and functional status of lymphocyte progenitor cells from patients with Fanconi’s anemia following x-irradiation. The relative radiosensitivity of lymphocytes from patients with Fanconi’s anemia was compared with those of clinically normal individuals, and the sensitivity of new assay methods for the quantitation of T-lymphocyte progenitors was determined.

MATERIALS AND METHODS

Case Histories

Fifteen clinically normal adults, 18–46 yr of age, were used as control subjects. Four Fanconi’s anemia patients were studied. The clinical profiles of these patients are summarized in Table 1.

Peripheral Blood

Peripheral blood was aseptically drawn into preservative-free sodium heparin (Calbiochem, San Diego, Calif.; 25 USP U/ml) and
mixed thoroughly. An aliquot of blood was removed for culturing; another aliquot was centrifuged, and the plasma removed.

**X-irradiation**

Whole blood was x-irradiated at 73 R/min, in equilibrium with air at 0 (sham-irradiated control), 12.5, 25, 50, 100, 200, and 400 R by a Maximar 250-III (General Electric, Milwaukee, Wisc.) unit.

**Colony Culture Methods**

Human T-lymphocyte colonies were grown, as recently described by Knox et al., using a modified method of Wilson et al. for the growth of canine T-lymphocyte colonies from whole blood. The whole blood colony technique eliminates the requirement for gradient-enriched lymphocyte fractions and provides a sensitive system for the study of T-lymphocyte progenitors that more closely approximates the in vivo milieu. Previous studies have shown that whole human blood colonies were composed of lymphoblasts and mature lymphocytes. Individual colony cells lacked lipase and specific esterase activity, were largely nonspecific, acid alphaphosphoryl acetate esterase (ANAE) positive, formed E rosettes, and were not phagocytic. Colony formation was also previously shown to increase as a power function over a wide range of cell concentrations. Maximal colony formation occurred when whole blood was plated at a final concentration of 3%. While studies to date have not clearly demonstrated the clonal origin of these colonies, the whole blood colony technique is nevertheless a very sensitive in vitro autologous plasma, and 25 µl/ml PHA-P (Difco Lab.), or 25 µg/ml PHA-P (Difco Labs., Detroit, Mich.), or 125 µg/ml Con-A (Calbiochem). One milliliter of the blood suspension was pipetted into each of 35 x 10 mm culture dishes (Corning Plastics, Corning, N.Y.). Cultures were plated in triplicate. The absolute numbers of white blood cells and lymphocytes plated in the whole blood colony technique using blood from Fanconi's anemia patients and clinically normal individuals are shown in Table 2. After incubation for 7 days at 37°C in 5% CO₂ humidified air atmosphere, the cultures were stained with new naphthyl acetate esterase (ANAE) positive, formed E rosettes, and were not phagocytic.

Whole blood was diluted to a final concentration of 3% in RPMI 1640 (Grand Island Biologic Co., Grand Island, N.Y.) containing 2 mM L-glutamine (GIBCO), 1% antibiotics-antimycotics (GIBCO), 10% autologous plasma, and 0.3% Sea-Plaque Agarose (Marine Colloids, Rockland, Me.). Lectins were added to the blood at a final concentration of 90 µg/ml PHA-P (Difco Laboratories, Detroit, Mich.), or 125 µg/ml Con-A (Calbiochem). One milliliter of the blood suspension was pipetted into each of 35 x 10 mm culture dishes (Corning Plastics, Corning, N.Y.). Cultures were plated in triplicate. The absolute numbers of white blood cells and lymphocytes plated in the whole blood colony technique using blood from Fanconi’s anemia patients and clinically normal individuals are shown in Table 2. After incubation for 7 days at 37°C in 5% CO₂ humidified air atmosphere, the cultures were stained with new Methylene Blue (0.25%) (Matheson, Coleman and Bell, Cincinnati, Ohio) at room temperature for 4 hr. Colonies were counted with a 40x dissecting scope, and only colonies comprised of more than 40 cells were scored.

**Lymphocyte Stimulation Test**

The whole blood lymphocyte stimulation microassay technique (WB/LST) developed for the canine was modified for human cells. Briefly, 50 µl of blood irradiated at different dose levels was added to 0.95 ml RPMI 1640 (GIBCO) containing 2 mM L-glutamine (GIBCO), 1% antibiotics-antimycotics (GIBCO), 10% autologous plasma, and 25 µg/ml PHA-P (Difco Lab.), or 25 µg/ml Con-A (Calbiochem). Cell suspensions were thoroughly mixed, and three 0.2-ml aliquots were pipetted into wells of a sterile flat-bottomed microtiter plate (Microbiological Assoc., Bethesda, Md.).
Radiosensitivity in Fanconi's Anemia

Patient and normal values for the absolute number of cells plated in the WB/LST are shown in Table 2. Plates were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Cultures were labeled with 1 μCi of tritiated thymidine (³HdTR; specific activity 6.7 Ci/m mole) (New England Nuclear, Boston, Mass.) per well in a 10-μl volume 18–20 hr before harvesting on a 6-well Skatron harvester (Flow Laboratories, Rockville, Md.). Samples were counted in a β-scintillation counter (Packard Instrument Co., Downers Grove, Ill.).

Statistical Methods

Radiation survival measurements were made in triplicate for each subject at each exposure level, and the arithmetic mean number of colonies or counts per minute (cpm) was calculated. For each individual, a survival curve was constructed by plotting the radiation dose against the log of the response, expressed as a percentage of the sham-irradiated control response. A least-squares linear regression analysis was applied to the exponential region of the survival curve, and the D₃₇ and slope (b) of the linear regression line were determined for each subject used in each test. The equation used was: percent survival, y = n e⁻ⁿᵇ, where n is the intercept at zero dose (extrapolation number), b is the slope, and D is the dose in R. The D₃₇ and b values for individuals within a test group (e.g., PHA WB/colonies) were used to estimate the mean and standard error of the mean (SEM) for these parameters.

Patient D₃₇ and b values were compared to those of normal subjects using the Mann-Whitney U test. Survival curves, D₃₇, and b values were determined for each test group by taking the mean of the surviving fractions for all test subjects at each dose and applying a least-squares linear regression analysis to the composite survival data.

RESULTS

The cloning efficiencies of lymphocytes from Fanconi's anemia patients and normal individuals are compared in Table 3. A marked increase (p = 0.08) relative to normal values was observed in the number of PHA-responsive circulating T-lymphocyte progenitors from the patient group. No significant differences in cloning efficiencies were observed with Con-A (p = 0.39).

Results of the dose-response experiments are summarized in Table 4. Normal control and Fanconi's anemia patient mean values for D₃₇ and slope (b) are presented. Individual values for the four patients are shown as well. D₃₇ and b values for patients nos. 1, 2, and 4 in the PHA WB/LST, and patients nos. 1, 3, and 4 in the PHA WB/colony assay, fell below the 95% confidence limits for the normal values. Extremely low D₃₇ values (11.7, 30.8, 55.6 R) were obtained for three of the patients in the WB/PHA colony assay. The relative radiosensitivity of patients 2

<p>| Table 3. Cloning Efficiencies Obtained for Peripheral Blood From Normal Subjects and Patients With Fanconi's Anemia |
|-----------------------------|-----------|-----------|-------------------------------|</p>
<table>
<thead>
<tr>
<th>Condition</th>
<th>Mitogen</th>
<th>n</th>
<th>Cloning Efficiency*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>PHA-P</td>
<td>21</td>
<td>187 ± 34</td>
</tr>
<tr>
<td>Fanconi's anemia</td>
<td>PHA-P</td>
<td>4</td>
<td>351 ± 125</td>
</tr>
<tr>
<td>Normal</td>
<td>Con-A</td>
<td>9</td>
<td>62 ± 22</td>
</tr>
<tr>
<td>Fanconi's anemia</td>
<td>Con-A</td>
<td>3</td>
<td>67 ± 44</td>
</tr>
</tbody>
</table>

Data presented as the mean ± SEM.

*The number of plated lymphocytes containing one progenitor cell (T-lymphocyte colony-forming unit [CFU-TL]) capable of forming one colony.

| Table 4. D₃₇ and Slope (b) Values of Survival Curves Obtained From Normal Subjects and Patients With Fanconi's Anemia Using ³HdTR Incorporation and T-Lymphocyte Colony Techniques |
|-----------------------------|-----------|-----------|-------------------------------|
| Fanconi's anemia patients   |           |           |                               |
| PHA-P                       |           |           |                               |
| Patient number              |           |           |                               |
| 1                           | 150.6     | -.00268   | 30.8 ± 0.0313                 |
| 2                           | 142.5     | -.00200   | 113.5 ± 0.0262                |
| 3                           | 308.2     | -.00136   | 55.6 ± 0.0470                 |
| 4                           | 191.0     | -.00240   | 11.7 ± 0.00479                |
| Mean ± SEM                  | 198.1 ± 38.2| -.00211 ± .00029| 52.9 ± 22.1| -.00381 ± .00055|
| p = .06                     | p = .04   | p = .02   | p = .12                       |
| Con-A                       |           |           |                               |
| Patient number              |           |           |                               |
| 2                           | 152.9     | -.00246   | 131.9 ± 0.0124                |
| 3                           | 232.1     | -.00183   | 94.2 ± 0.0239                 |
| 4                           | 302.7     | -.00156   | 93.7 ± 0.0314                 |
| Mean ± SEM                  | 229.2 ± 43.3| -.00195 ± .00027| 106.6 ± 12.7| -.00226 ± .00055|
| p = .12                     | p = .14   | p = .31   | p = .40                       |
| Normal values (mean ± SEM)  |           |           |                               |
| PHA-P                       | 308.5 ± 29.3| -.00156 ± .00016| 109.3 ± 9.5| -.00331 ± .00026|
| Con-A                       | 266.7 ± 16.1| -.00167 ± .00012| 110.2 ± 13.4| -.00280 ± .00014|

*p Values were obtained for comparisons of Fanconi's anemia patient values with normal values using the Mann-Whitney U test.
and 3 was inconsistent in the two assays. Colony formation was more sensitive to radiation effects than the LST with both patient and control cells. The magnitude of difference in sensitivity of the two methods was approximately equivalent in both groups.

The patient mean values for the PHA WB/LST ($b$, $p = 0.04; D_{37}, p = 0.06$) and PHA WB/colony assay ($D_{37}, p = 0.02$) were significantly different from the means of the control group. There were no significant differences in either the $D_{37}$ or $b$ values between the four Fanconi's anemia patients relative to normals for Con-A using either the WB/LST or colony assay.

Survival curves, $D_{37}$, and $b$ values were determined for each test group by taking the mean of the surviving fractions for all test subjects at each dose and applying a least-squares linear regression analysis to the composite survival data. The linear regression lines for the exponential portion of the survival curves are shown in Fig. 1. Correlation coefficients for the least square fits ranged between 0.96 and 0.99. Values for the extrapolation number, $n$, were approximately equal to 1.0 for both patient and control groups.

**DISCUSSION**

Lymphocytes from patients with Fanconi's anemia were significantly more sensitive to in vitro x-irradiation than lymphocytes from clinically normal individuals, as measured by their ability to divide following stimulation by PHA in the LST and colony formation assay. In contrast, no significant differences in the relative radiosensitivity of Con-A-responsive lymphocytes were observed for either test.

![Fig. 1. Linear regression lines ($y = ne^{ax}$) for the exponential portion of the radiation survival curves for lymphocytes from normal subjects and patients with Fanconi's anemia, stimulated by (A) PHA in the whole blood colony assay, (B) Con-A in the whole blood colony assay, (C) PHA in the whole blood LST, (D) Con-A in the whole blood LST. The error bars represent mean ± SEM.](image-url)
This dichotomous response of PHA and Con-A subpopulations in Fanconi's anemia patients was also reflected in the cloning efficiencies. A significant increase above normal values ($p = 0.08$) was observed in patients for PHA- but not Con-A-responsive T-lymphocyte progenitors ($p = 0.39$). PHA- and Con-A-responsive lymphocytes appear to be independently affected in Fanconi's anemia. Our results are in agreement with previous studies demonstrating that different subpopulations of T lymphocytes react differently to Con-A and PHA. In the canine we have also obtained evidence that Con-A and PHA stimulate different subpopulations of lymphocytes with different radiosensitivities.

The basis of the increased cellular radiosensitivity of Fanconi's anemia patients is unknown. Fanconi's anemia patients reportedly have defective mechanisms of DNA repair. Ineffective repair of crosslinked DNA and defective exonuclease activity involved in the excision of DNA lesions have been reported. Decreased intracellular levels of the radioprotective enzyme superoxide dismutase (SOD) have been observed in erythrocytes of patients with Fanconi's anemia. The mean level of SOD in the erythrocytes of the patients used in this study was approximately one-third ($p = 0.02$) that of the controls (Knox et al., unpublished results).

SOD is a scavenger of the superoxide radical, $O_2^-$, produced as a result of radiolysis of water by ionizing radiation. $O_2^-$ has been demonstrated to damage chromosomes. It is possible that decreased levels of intracellular SOD play a role in the increased spontaneous occurrence of chromosomal aberrations and radiosensitivity of cells from patients with Fanconi's anemia. Exogenous addition of SOD has been reported to decrease the frequency of chromosomal breaks in Fanconi's anemia cells.

The increased radiosensitivity and cloning efficiency of PHA-responsive lymphocytes from patients with Fanconi's anemia is suggestive of an intrinsically defective T-lymphocyte subpopulation. This is of interest as pancytopenia occurring in Fanconi's anemia has been attributed to a defect at the level of the pluripotent hematopoietic stem cell, as indicated by successful responses to bone marrow reconstitution. Fanconi's anemia patients commonly have markedly decreased numbers of granulocyte-monocyte and erythroid progenitor cells. As previously reported, on two separate occasions, granulopoietic colonies could not be grown from the bone marrow cells of patient no. 1. It is possible that abnormalities in lymphoid regulator cells for hematopoiesis result in defective cellular interactions that may be indirectly responsible for the resultant bone marrow failure.

Fanconi's anemia patients and their families have an increased incidence of leukemia and solid malignant tumors. It is well known that genetic and environmental factors may predispose some humans to radiation-induced malignancies. While the incidence of Fanconi's anemia is very low, the heterozygous condition is approximately 1200 times as prevalent.

In a preliminary experiment, in vitro radiation survival of lymphocytes from the parents of patient 1 were studied. The father showed a twofold increase in radiosensitivity in the WB/colony and LST assays, while the mother was within the normal range.

These studies are germane to the assessment of the relative radiosensitivity and associated risk of different population groups and are required for the establishment of conservative and prudent radiation protection standards. The WB/LST and colony formation assays provide sensitive in vitro bioassays for the determination of the relative radiosensitivity of individuals with genetic abnormalities in lymphohematopoiesis and potentially increased susceptibility to radiation-associated carcinogenesis.

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REFERENCES


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