Damaged DNA in Lymphocytes of Aplastic Anemia

By Yasuo Hashimoto, Fumimaro Takaku, and Kinori Kosaka

The size of single-stranded DNA in lymphocytes in G_0 stage from 22 patients with acquired primary and secondary aplastic anemia was estimated by alkaline sucrose gradient centrifugation. The average size was 9.3 ($\pm$0.3) $\times$ 10^7 daltons. The lymphocytes of patients contained significantly more single-strand breaks in DNA, compared to those of normal persons. The difference in size of single-stranded DNA which had been present in nontransformed lymphocytes could also be observed in transformed lymphocytes. Some characteristic differences could be observed in the sedimentation patterns of single-stranded DNA in the lymphocytes of patients with aplastic anemia and those of normal persons. The single-strand breaks in DNA suggested that the repair processes were disturbed in the DNA molecules of circulating lymphocytes from patients with acquired primary and secondary aplastic anemia.

CHROMOSOMAL BREAKAGE is frequently associated with malignancy and may be found as the harbinger of malignancy in disorders such as the Fanconi syndrome.[1] Disturbed DNA repair has been suggested as the cause of chromosomal breakage in the Fanconi syndrome.[17]

A new method for the quantitative estimation of the size of single-stranded DNA of somatic cells in an animal without using isotopes has been developed. The technique involves a fluorescent microassay of DNA subjected to alkaline sucrose centrifugation.[7-9]

A somatic cell may possess single-stranded DNA of a unique size and the enzymatic machinery needed to repair single-strand breaks in DNA.[2,8,9,16] The size of single-stranded DNA in circulating lymphocytes of patients with Fanconi syndrome has been measured and found to be smaller than that of normal persons, whether or not chromosomal breakage could be found.[3] The smaller size of the single-stranded DNA suggested an increase of single-stranded breaks in DNA and the existence of a disturbed process of DNA repair. In the present study, we have searched for a similar increase in single-strand breaks in the DNA of lymphocytes from patients with acquired aplastic anemia.

MATERIALS AND METHODS

Radioactive Labeling of Murine Leukemic Cells (L5178Y)

L5178Y cells were maintained in Fisher's medium supplemented with 10% horse serum (Chiba Kessei Institute, Chiba, Japan). The exponentially growing populations were labeled for 20 hr with ^{3}H-thymidine (0.5 μCi/ml, 5.0 Ci/mmole, The Radiochemical Centre Ltd., Amersham,

From The Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo, Japan and the First Department of Medicine, Jichi Medical School, Tochigi, Japan.

Submitted March 5, 1974; accepted May 2, 1975.

Supported in part by research grants for specific diseases from the Ministry of Health and the Welfare Ministry, Japan.

Address for reprint requests: Yasuo Hashimoto, M.D., The Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo, Japan 113.

Table 1. Hematologic Data of Patients With Aplastic Anemia

Patients No.	Name	Sex	Age	Hb (g/dl)	RBC ($\times 10^4$/ cu mm)	WBC (per/ cu mm)	Ht. (%)	Platelet ($\times 10^4$/ cu mm)	Retics ($/10^3$ RBC)	Prednine (mg/day)	Suggestive Agents
1.	A.I.	Male	19	6.0	151	2400	17.4	3.3	21	10	Unknown
2.	K.K.	Male	37	7.3	207	3000	20.8	1.8	19	30	Unknown
3.	F.W.	Female	53	6.5	172	2900	18.6	0.4	32	30	Unknown
4.	M.I.	Male	28	8.5	278	2400	24.6	1.2	—	30	Stilene monomer
5.	Y.M.	Male	28	14.5	391	4300	41.1	7.1	24	—	Unknown
6.	M.S.	Male	61	11.1	314	4300	33.1	5.2	18	5	Chloramphenicol
7.	K.M.	Female	21	6.1	166	1800	17.5	0.5	16	30	Unknown
8.	T.H.	Male	28	7.4	214	2100	20.2	2.4	22	30	Unknown
9.	H.O.	Female	56	10.6	267	2800	30.9	4.3	37	30	Hair dye
10.	S.Y.	Male	27	5.1	122	3500	15.3	0.2	35	30	Unknown
11.	Y.M.	Female	10	4.4	101	2500	12.6	1.7	88	30	Unknown
12.	M.K.	Female	21	6.0	158	3100	17.4	3.0	48	30	Unknown
13.	A.M.	Female	35	12.1	331	2900	38.4	5.0	43	—	Unknown
14.	Y.M.	Female	68	9.5	255	2700	32.0	3.6	20	—	Unknown
15.	T.K.	Female	43	8.0	203	1900	23.4	2.4	—	10	Unknown
16.	M.U.	Male	20	9.5	219	2600	34.0	0.8	7	10	Unknown
17.	H.T.	Male	11	8.1	207	3200	25.0	8.2	9	—	Unknown
18.	K.A.	Female	46	7.8	290	8700	22.0	3.0	33	30	Unknown
19.	Z.I.	Male	50	6.9	196	3500	23.0	3.0	40	—	Unknown
20.	S.U.	Female	68	6.7	187	2300	19.0	1.0	56	—	Unknown
21.	K.K.	Male	46	5.9	159	2200	19.3	1.0	31	—	Unknown
22.	J.K.	Female	40	11.0	326	4100	34.7	8.3	40	—	Unknown

England). The cells were spun down, resuspended in medium TC199, and subjected to sedimentation analysis. The specific activity of 0.2 ml of each fraction was counted in the usual manner, and the DNA content of each fraction was estimated with fluorescence.

Patients and Normal Subjects

The diagnosis of aplastic anemia was based on accepted clinical and laboratory criteria, including pancytopenia in peripheral blood, bone marrow aplasia, elevated serum iron concentration, and other laboratory findings indicating the presence of decreased hematopoiesis. Hematologic data of these patients are summarized in Table 1. Suggestive agents that may have caused the aplastic anemia are included in Table 1. Normal controls were laboratory personnel.

Lymphocyte Preparation

Lymphocytes were separated from the peripheral blood of the patients using Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden) and Conray (Mallinckrodt Chemical Works, St. Louis, Mo.) according to the method of Böyum.[4] Two-milliliter aliquots of heparinized blood were layered over 3 ml of a solution composed of 25 parts of 10% Ficoll, 6 parts of Conray, and 4.3 parts of Tyrode's solution. The samples were centrifuged at 0°C (800 *g*, 20 min). The lymphocytes at the interphase were aspirated with a Pasteur pipette, washed twice with Tyrode's solution, centrifuged at 600 *g* for 5 min, and resuspended in medium TC199 at a concentration of approximately 2×10^6 cells per ml. Lymphocyte preparations were employed only if their purity was 95% or greater, and the separations were performed at 0°C to exclude endonuclease action against DNA strands.

Cell Culture and ^{3}H-thymidine Labeling

For culture of lymphocytes, cells were resuspended in Eagle's medium with 10% fetal calf serum (Gibco, Berkeley, Calif.), 200 U penicillin-G per ml, 200 μg streptomycin sulfate per ml, and 10 μg per ml of phytohemoagglutinin-P (Gibco). The cells were cultured at 37°C for 68 hr, and ^{3}H-thymidine (1 μCi/ml, 5.0 Ci/mmole, The Radiochemical Centre Ltd.) was added for the last

20 hr of the incubation. The cells were spun down, resuspended in medium TC199, and subjected to sedimentation analysis.

Centrifugation

Alkaline sucrose gradients were prepared as described by Lett et al.[5] One-half milliliter of cell suspension (about 1×10^6 cells) and 0.5 ml of lysing solution (0.5 *N* NaOH and 0.1 *M* Na_2EDTA) were gently layered on top of 30 ml of the gradient (5%–20%) and kept at 29°C for 3 hr to let the cells lyse and to denature double-stranded DNA molecules into single-stranded DNA. The gradients were centrifuged in a Hitachi model 55P ultracentrifuge using a RPS-25A rotor (Rav. 7.3 cm) at 24,000 rpm for 210 min at 12°C. The gradient was fractionated every 2 ml from its top by introducing 50% sucrose solution into the bottom of the centrifuged tube. To each fraction, 0.1 ml of 1 mg/ml bovine serum albumin (Miles Laboratories, Indiana) and 2 ml of 10% trichloroacetic acid were added. After storage overnight at 4°C, the samples were centrifuged at 1200 *g* for 30 min in order to coprecipitate DNA with bovine serum albumin. The precipitates were washed twice with 5% trichloroacetic acid and once with 0.1 *M* potassium acetate in alcohol to remove sucrose and lipid. Finally, 2 ml of absolute alcohol were added to each precipitate which was kept in a waterbath at 60°C for 15 min. After centrifugation, the supernatants were removed by aspiration, and the precipitates were dried. Fifty microliters of 2 *M* 3,5-diaminobenzoic acid dihydrochloride was added to each dried sample. The preparations were kept in a waterbath at 60°C for 45 min. Finally, 3 ml of 0.6 *N* perchloric acid were added to each sample. The fluorescence of each sample at 510 nm with excitation of 414 nm, specific for the deoxyribose moiety of DNA, was determined with a fluorospectrophotometer model RF 502, Shimazu Co., Ltd.

Table 2. Number Average Molecular Weight of Single-stranded DNA in Lymphocytes From Patients With Aplastic Anemia and Normal Persons

Control No.	Mn ($\times 10^8$) Mean ± SE	Patient No.	Mn ($\times 10^8$) Mean ± SE
1	1.2 ± 0.04	1	0.95 ± 0.01*
2	1.4 ± 0.03	2	0.91 ± 0.06*
3	1.3 ± 0.2	3	0.98 ± 0.05*
4	1.3 ± 0.04	4	0.89 ± 0.05*
5	1.0 ± 0.01	5	0.91 ± 0.05*
6	1.3 ± 0.03	6	0.95 ± 0.06*
7	1.3 ± 0.10	7	0.88 ± 0.04*
8	1.2 ± 0.04	8	0.94 ± 0.03*
9	1.3 ± 0.04	9	1.02 ± 0.08*
10	1.0 ± 0.02	10	1.04 ± 0.11*
11	1.3 ± 0.06	11	1.16 ± 0.06
12	1.4 ± 0.03	12	1.24 ± 0.01
13	1.3 ± 0.07	13	0.85 ± 0.04*
14	1.2 ± 0.05	14	0.88 ± 0.02*
15	1.3 ± 0.07	15	0.86 ± 0.02*
16	1.0 ± 0.06	16	0.96 ± 0.07*
17	1.1 ± 0.06	17	0.75 ± 0.07*
18	1.2 ± 0.03	18	0.61 ± 0.05*
19	1.3 ± 0.06	19	0.94 ± 0.07*
20	1.1 ± 0.04	20	0.89 ± 0.07*
		21	1.02 ± 0.05*
		22	0.93 ± 0.04*

Student's t test was performed between patients and normal persons. The statistical definition is described in Materials and Methods. SE, standard error. Patient Number corresponds to that given in Table 1. Mn, number average molecular weight.

*Significant ($p < 0.05$).

Estimation of Molecular Weight of Fractionated DNA

T4 and λ bacteriophage with molecular weights of 6.5×10^7 and 1.7×10^7 daltons, respectively, were centrifuged on the same sucrose gradient in order to determine the two standards (α,β) in Burgi's equation: $D = \alpha \cdot M^{\beta}$.[6] The weight average molecular weight of the applied DNA was determined by calculating the number of single-stranded DNA molecules in each fraction and the molecular weight of each fraction. The half-value of the weight average molecular weight was taken to represent the number average molecular weight.[14]

Statistical Considerations

For statistical analysis, the patients with aplastic anemia were compared to normal persons. For comparisons between means, Student's t test was used with $(N_1 - 1) + (N_2 - 1)$ degrees of freedom, where N_1 and N_2 were the numbers of observations in each patients or all patients with aplastic anemia and all normal persons, respectively (Table 2). A p value of less than 0.05 was considered significant. The standard error was added to the value, instead of the standard deviation.

RESULTS

Confirmation of Method With Radioactivity and Fluorescence

No remarkable difference was noted between the two patterns in Fig. 1.[7-9] The number average molecular weight of the former was 1.06×10^8 daltons and the latter 1.14×10^8 daltons. The recovery of applied DNA was about 80% with a fluorometric estimation.

Sedimentation Profiles and Size of Single-stranded DNA of Lymphocytes From Normal Persons

As shown in Fig. 2, the number average molecular weight was (1) 1.2×10^8 daltons, (11) 1.3×10^8 daltons, (13) 1.3×10^8 daltons, and (14) 1.2×10^8 daltons, respectively. The single main peak was observed in fraction 11 or 12 in each profile, accompanied by a gentle slope to both sides. Neither a shoulder nor an accessory peak was observed in the DNA profiles of normal persons. The values of the size of single-stranded DNA in lymphocytes from normal persons are presented in Table 2, with a mean value of $1.23 (\pm 0.03) \times 10^8$ daltons.

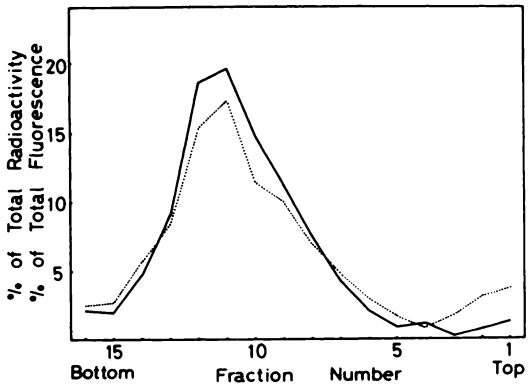

Fig. 1. Sedimentation patterns of single-stranded DNA in murine leukemic cells (L5178Y). ———, the pattern, estimated with fluorescence. The number average molecular weight is 1.06×10^8 daltons. ....., the pattern, estimated with radiosensitivity. The number average molecular weight is 1.14×10^8 daltons.

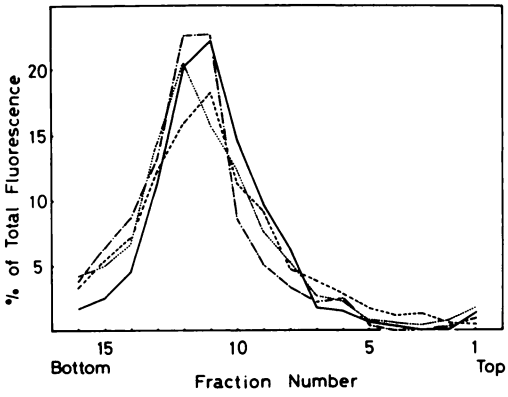

Fig. 2. Sedimentation patterns of single-stranded DNA in lymphocytes in G_0 stage from normal persons, estimated with fluorescence. ————, control No. 1. The number average molecular weight is 1.2 × 10^8 daltons. —·—·—·–, control No. 11. The number average molecular weight is 1.3 × 10^8 daltons. - - - - - - - -, control No. 13. The number average molecular weight is 1.3 × 10^8 daltons. ....., control No. 14. The number average molecular weight is 1.2 × 10^8 daltons.

Sedimentation Profiles and Size of Single-stranded DNA in Lymphocytes From Patients With Aplastic Anemia

The main peak was slightly shifted to the right in these patterns, as compared to those of normal persons (Fig. 3). A definite shoulder could be observed (patient Nos. 15 and 19). The number average molecular weight of single-stranded DNA in lymphocytes from patients with aplastic anemia is shown in Table 2, and the mean size of the single-stranded DNA is significantly smaller than normal values, except in two instances. The mean value of number average molecular weight was 0.93 (±0.03) × 10^8 daltons.

Sedimentation Profiles and Size of Single-stranded DNA in Transformed Lymphocytes

Marked differences were seen in the size of single-stranded DNA and in the sedimentation profile between patients with aplastic anemia and normal persons (Table 3). Characteristically, the sedimentation patterns of single-stranded DNA in lymphocytes of patients with aplastic anemia showed many small peaks from the top to fraction 10, suggesting that the degraded DNA was accentuated in transformed lymphocytes. The average size of single-stranded DNA of transformed lymphocytes from patients with aplastic anemia and from normal persons was 0.85 (±0.06) × 10^8 daltons and 1.18 (±0.04) × 10^8 daltons, respectively (Fig. 4).

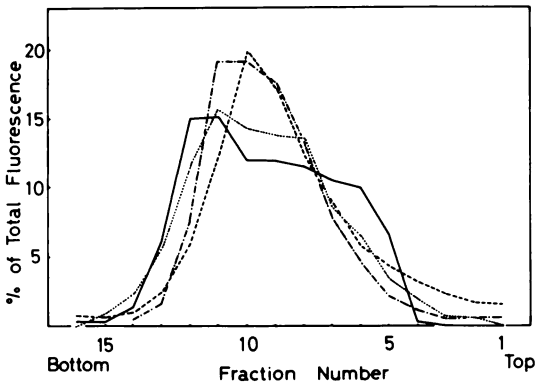

Fig. 3. Sedimentation patterns of single-stranded DNA in lymphocytes in G_0 stage from patients with aplastic anemia, estimated with fluorescence. ————, patient No. 19. The number average molecular weight is 0.89 × 10^8 daltons. — - — - — - –, patient No. 14. The number average molecular weight is 0.89 × 10^8 daltons. ----------, patient No. 13. The number average molecular weight is 0.78 × 10^8 daltons. ....., patient No. 15. The number average molecular weight is 0.91 × 10^8 daltons.

Table 3. Size of Single-stranded DNA in Transformed Lymphocytes From Patients With Aplastic Anemia and Normal Persons

Control Number	Mn ($\times 10^8$) Mean ± SE	Patient Number	Mn ($\times 10^8$) Mean ± SE
1	1.3 ± 0.1	6	1.0 ± 0.05
2	1.1 ± 0.1	7	0.92 ± 0.07*
9	1.1 ± 0.1	14	0.90 ± 0.11*
11	1.2 ± 0.04	15	0.70 ± 0.06*
15	1.2 ± 0.1	19	0.73 ± 0.04*

Mn, number average molecular weight. Student's t test was performed between patients and normal persons. Patient Number and Control Number correspond to those given in Tables 1 and 2. The statistical definition is described in Materials and Methods. SE, standard error.

*Significant ($p < 0.05$).

DISCUSSION

In our previous study,[2] the number average molecular weight of single-stranded DNA in peripheral lymphocytes from normal persons was 1.1 × 10^8 daltons and, in the present study, 1.2 × 10^8 daltons. This discrepancy was considered to be due to an alteration in the DNA standards (T4 and λ bacteriophage). The difference in sedimentation patterns and mean size of single-stranded DNA in lymphocytes between normal persons and patients with aplastic anemia suggested that the peripheral lymphocytes in the latter did not represent a single cell population.

The results obtained with stimulated lymphocytes from patients with aplastic anemia suggested the following possibilities: (1) The damaged DNA of resting lymphocytes was transmitted to and exaggerated in blastic lymphocytes; (2) The DNA breaks existed in T-cells, but it is not for certain in B-cells, thereby raising the possibility of a role of T-cells in bone marrow failure. The single-strand breaks in DNA might have been due to: (1) Mechanical damage during cell preparation. The Ficoll-Conray method permits the preparation of normally functioning lymphocytes for experiments in immunology. The possibility still remains, however, that fragile lymphocytes would be susceptible to DNA breaks during cell preparation. (2) Endonuclease action on DNA strands during cell preparation. The lymphocytes, however, were separated at 0° C to prevent

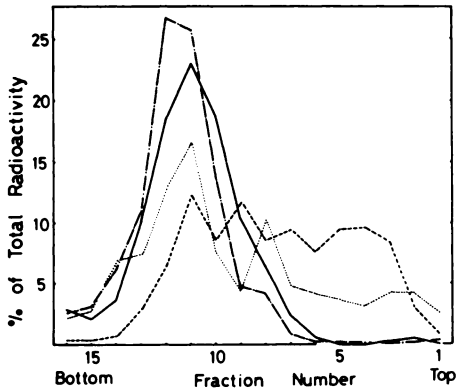

Fig. 4. Sedimentation patterns of single-stranded DNA in transformed lymphocytes from patients with aplastic anemia and normal persons, estimated with radiosensitivity. ——————, control No. 15. The number average molecular weight is 1.2 × 10^8 daltons. —·—·—·—, control No. 1. The number average molecular weight is 1.3 × 10^8 daltons. - - - - - - - - - - - -, patient No. 15. The number average molecular weight is 0.65 × 10^8 daltons. . . . . , patient No. 6. The number average molecular weight is 1.0 × 10^8 daltons.

endonuclease action. (3) Longer cell lysis time may have caused more single-strand breaks in the DNA of somatic cells. Lett, however, provided evidence that the mean size of single-stranded DNA did not change even if the lysing time was increased from 3 to 6 hr.[5] (4) Small-sized single-stranded DNA could not be obtained from lymphocytes of patients with a collagen disease who had received large doses of prednisolone for more than 4 wk. These considerations suggested that damaged single-stranded DNA (alkaline labile bonds) was present in lymphocytes in vivo in patients with aplastic anemia.

The accumulation of DNA breaks in mammalian cells has been considered to be a consequence of the aging process.[13] We would like to propose that the peripheral lymphocytes from patients with aplastic anemia might be aged somatic cells because normal lymphocytes are still able to repair DNA damages. Chloramphenicol, however, is one of the causative agents of aplastic anemia, and it has been reported that this agent inhibits DNA repair,[10] while cycloheximide inhibits chromosomal repair.[15] Although the pathophysiologic significance of the presence of single-strand breaks in DNA of somatic cells in patients with aplastic anemia is not clear at this moment, it may be related to the well-known fact that some patients with aplastic anemia terminate with acute leukemia. Human mutant syndromes, associated with some defects in the repair system for DNA, also tend to accompany cancer.[11,12]

ACKNOWLEDGMENT

The authors wish to thank Dr. S. Okada, Dr. N. Suzuki, and Dr. T. Ono for technical advice and for providing us with murine leukemic cells. We are grateful to Dr. Wakabayashi, Dr. Omine, Dr. Maekawa, and Dr. Miyazaki for providing us with blood from patients with aplastic anemia.

REFERENCES

1. Bloom G, Warner S, Gerald P, Diamond L: Chromosome abnormalities in constitutional aplastic anemia. N Engl J Med 274:8, 1966

2. Hashimoto Y, Ono T, Okada S: Radiosensitivities of DNA molecules in lymphocytes from the circulating blood of man. Blood 45: 503, 1975

3. Hashimoto Y, Takaku F, Higurashi M, Kosaka K: Sedimentation analysis of DNA in circulating lymphocytes from patients with Fanconi syndrome. (in preparation)

4. Böyum A: Separation of leukocytes from blood and bone marrow. Scand J Clin Lab Invest (Suppl) 97:21, 1968

5. Lett JT, Klucis ES, Sun C: On the size of the DNA in the mammalian chromosome: structural subunits. Biophys J 10:277, 1970

6. Burgi E, Hershey AD: Sedimentation rate as a measure of molecular weight of DNA. Biophys J 3:309, 1963

7. Ono T, Okada S: An alkaline sucrose gradient centrifugation method applicable to non-dividing and slowly dividing cells. J Radiat Res (Tokyo) 14:42, 1973

8. Karran P, Ormerod M: Is the ability to repair damages to DNA related to the proliferative capacity of a cell? The rejoining of x-ray-produced strand breaks. Biochim Biophys Acta 299:54, 1973

9. Wheeler KT, Lett JT: Formation and rejoining of DNA strand breaks in irradiated neurons: in vivo. Radiat Res 52:59, 1972

10. Bridges BA: Evidence for a further dark repair process in bacteria. Nature (New Biol) 240:52, 1972

11. Cleaver JE: Defective repair replication of DNA in xeroderma pigmentosum. Nature 218:652, 1968

12. Epstein J, Williams JR, Little JB: Deficient DNA repair in human progeroid cells. Proc Natl Acad Sci USA 70:977, 1973

13. Price GB, Modak SP, Makinodan T: Age-associated changes in the DNA of mouse tissue. Science 171:917, 1971

14. Charlesby A: Molecular-weight changes in the degradation of long-chain polymers. Proc R Soc Ser A 224:120, 1967

15. Wolf S: The repair of X-induced chromosome aberrations in stimulated and unstimulated human lymphocytes. Mutat Res 15:435, 1972

16. Ono T, Okada S: Estimation in vivo of DNA strand breaks and their rejoining in thymus and liver of mouse. Int J Radiat Biol 25:291, 1974

17. Sasaki MS, Tonomura A: A high sensitivity of Fanconi's anemia to chromosome breakage by DNA cross-linking agents. Cancer Res 33:1829, 1973