Jpn. J. Cancer Res. 85, 610–618, June 1994

Increased Rate of Spontaneous Mitotic Recombination in T Lymphocytes from a Bloom's Syndrome Patient Using a Flow-cytometric Assay at *HLA-A* Locus

Yoichiro Kusunoki,[1] Tomonori Hayashi,[1] Yuko Hirai,[1] Jun-ichi Kushiro,[1] Kouichi Tatsumi,[2] Takayuki Kurihara,[2,3] Mohamed Zghal,[4] Mohamed R. Kamoun,[4] Hiraku Takebe,[2,5] Alec Jeffreys,[6] Nori Nakamura[1] and Mitoshi Akiyama[1,7]

[1]*Department of Radiobiology, Radiation Effects Research Foundation, 5-2 Hijiyama Park, Minami-ku, Hiroshima 732,* [2]*Department of Molecular Oncology, Faculty of Medicine, Kyoto University, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606,* [3]*Medical Research Institute, Kanazawa Medical University, 1-1 Daigaku, Uchinada-cho, Kawakita-gun, Ishikawa 920-02,* [4]*Department of Dermatology, Charles Nicolle Hospital, 1006, Tunis, Tunisia,* [5]*Department of Experimental Radiology, Faculty of Medicine, Kyoto University, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606 and* [6]*Department of Genetics, University of Leicester, University Road, Leicester, UK*

Bloom's syndrome (BS) is an autosomal recessive disorder conferring high propensity for cancer and displaying a high degree of genetic instability; the frequency of sister chromatid exchange is characteristically 10 times above background. The symmetrical four-armed chromatid interchanges are much more readily detected in peripheral blood lymphocytes of BS patients, suggesting that the frequency of somatic recombination is also increased. In the present study, the rate of spontaneous loss of *HLA-A* allele expression was estimated following fluctuation analysis in cultured T lymphocytes using a flow-cytometric assay. It was found to be 10 times or more higher than normal in lymphocytes from a BS patient. Molecular and chromosome analyses showed that all 13 independent variants from the patient were most likely derived from somatic recombinations. Further tests for loss of heterozygosity at a closely linked proximal locus, *HLA-DQA1*, showed that as many as half of the recombinants retained heterozygosity irrespective of the donor. The results suggest that the HLA region is hyperrecombinogenic in somatic cells and that the elevated recombination rate in BS cells results from the general increase at ordinary sites and not from random creation of unusual sites for recombination.

Key words: Bloom's syndrome — *HLA-A* locus — Mitotic recombination — Somatic mutation — Spontaneous mutation rate

Bloom's syndrome (BS) is an autosomal recessive disorder first described in 1954 by Bloom.[1)] BS patients are characterized by short stature, congenital telangiectatic erythema, disrupted immune function and, among other defects, a high propensity for cancer.[1,2)] With the advent of cytological techniques, chromosome analysis of peripheral blood lymphocytes revealed the presence of rare symmetrical four-armed chromatid interchanges (Qrs).[3,4)] This was an important criterion for the diagnosis of BS until sister chromatid exchange (SCE) frequency was also found to be elevated by a factor of about 10 in BS patients.[5)]

Several theories have been proposed to explain the primary ethiology of the disease, namely, alterations in DNA ligase I,[6–8)] in topoisomerase II,[9)] in DNA glycosylase,[10)] in thymidylate synthetase,[11)] or in superoxide dismutase.[12)] However, the most recent results, using a minicell fusion technique, suggested that only human chromosome 15q14-qter can complement the elevated SCE frequency of BS cells[13)] and argued against all the above-mentioned theories as the primary cause of the disease, since none of the genes suspected above is located in chromosome 15q.

The rate of spontaneous mutations for BS-derived cultured cells *in vitro*[14,15)] and the mutant frequency of BS patients' lymphocytes and erythrocytes *in vivo*[16–18)] are known to be elevated. Increased frequencies of Qrs are regarded as supporting evidence for the elevated somatic recombination frequency in BS cells. In fact, erythrocyte mutation studies *in vivo* suggest an increased frequency of possible recombination types,[17,18)] although no molecular confirmation could be achieved because these cells lack DNA.

In the present study, monoclonally propagated T-lymphocyte cultures were established from one BS patient and three normal donors, and the rate of the spontaneous loss of expression of one *HLA-A* allele was estimated following fluctuation analysis using a recently developed flow-cytometric assay.[19)] Further, variant colonies lack-

[7] To whom correspondence should be addressed.

ing the expression of *HLA-A2* allele were established and analyzed for molecular and chromosomal changes.

MATERIALS AND METHODS

Blood donors Peripheral blood was obtained from one female Tunisian BS patient whose parents are cousins and three normal Japanese males aged 48, 34, and 30 years, i.e., donors 1, 2, and 3, respectively. The patient was 3 years old at the time of blood sampling. At birth her weight was 2.25 kg, height 48 cm, and head circumference 35 cm; at age 5, her weight was 11 kg, height 95 cm, and head circumference 45 cm, which were all about 3 standard deviations below average. She has skin erythema and telangietasia, "lupus-like" facial appearance, and several abdominal cafe-au-lait spots. Plasma IgM (60 mg/100 ml) and IgG (650 mg/100 ml) levels were normal, but IgA level was subnormal (122 mg/100 ml). She is of normal intelligence, with X-ray and CT scans showing only microcephaly. On the basis of clinical information and laboratory data on SCE frequency in lymphocytes (see "Results"), she was diagnosed as suffering from Bloom's syndrome. The patient was typed as *HLA-A2, w36; B12,*−; *Cw6,*−; *BW4,6* by the standard NIH method.

Lymphocyte culture Peripheral blood mononuclear cells (PBMCs) were isolated as described previously.[19] Because the blood sample from the BS patient was obtained 5 days after the blood was drawn, the isolated PBMCs showed decreased levels of HLA-A2 and CD4 antigens on the surface, and the modal peak was about one half of that usually observed for fresh PBMCs (not shown).

For the initial mass culture, 2×10^4 PBMCs were seeded in each well of a 24-well plate (total 10 wells) with feeder cells (5×10^5 allogeneic PBMCs and 10^5 lymphoblastoid cells, OKIB, irradiated with 50 Gy and 100 Gy of X rays, respectively). The culture medium consisted of GIT-medium (a serum-free medium specified for hybridoma cells; Wako Pure Chemicals) supplemented with 10% fetal calf serum, 1:6400 phytohemagglutinin-P (PHA) (Difco Lab.) and 2 ng/ml human recombinant interleukin-2 (IL-2) (a gift from Takeda Chemical Industries, Ltd.). The mass cultured BS cell line was maintained by dilution at appropriate intervals and by weekly refeeding with the feeder cells.

As for the cloning to prepare monoclonal cultures for the fluctuation test, cells from the mass culture of the BS patient at 2 weeks after culture or PBMCs from the normal donors were distributed into round-bottomed 96-well microtest plates (Costar) at a mean frequency of 0.5 cell per well using the same medium described above but one-fifth the number of feeder cells used for the 24-well plates. After about 2 weeks, each growing colony was transferred into a well of a 24-well plate, and feeder cells were added, followed by a 1:3 split without feeder cells for subsequent measurement of variant frequency by flow cytometry (at least 1 week after addition of feeder cells).

Monoclonal antibodies (mAbs) Fluorescein isothiocyanate (FITC)-labeled anti-Leu-4 (CD3), anti-Leu-3a (CD4), anti-Leu-2a (CD8), and anti-TCR1 (TCR$\alpha\beta$) mAbs and phycoerythrin (PE)-labeled anti-Leu-4 (CD3) and anti-Leu-3a (CD4) mAbs were obtained from Becton-Dickinson Immunocytometry Systems. FITC-labeled anti-TCR-δ1 (TCRδ) mAb was purchased from T-Cell Diagnostics Inc. Biotin-labeled anti-HLA-A2 mAb was prepared as described previously.[19]

Flow cytometry Approximately 10^6 cells were incubated with 10 μl of biotin-labeled anti-HLA-A2 and 20 μl of FITC-labeled anti-Leu-4 mAbs, each at a concentration of 25 μg/ml for 30 min on ice. After being washed with 0.015 *M* phosphate-buffered saline (PBS, pH 7.4), the cells were resuspended in 40 μl of PE-labeled streptavidin (Becton-Dickinson) and kept on ice for 30 min. The cells were washed again and were resuspended in PBS containing 10 μg/ml propidium iodide before being subjected to flow cytometry. About 2×10^5 cells were analyzed using a FACScan (Becton-Dickinson), and the frequency of $CD3^+$ T cells lacking *HLA-A2* expression was measured. Feeder cell contamination in the variant window was estimated to be below 2×10^{-5} (0, 0, 0, 0.5×10^{-5} and 2×10^{-5} for five cultures, respectively) by testing 2×10^5 *HLA-A2* homozygous cells per culture 1 week after seeding with feeder cells which did not bear *HLA-A2* allele.

Cell-surface expression of CD3, CD4, CD8, TCR$\alpha\beta$, and TCR$\gamma\delta$ in the clonal T cells was analyzed as reported previously.[20]

Preparation of HLA-A2$^-$ variant clones Cells from the T-cell mass culture at 4 weeks after culture and two monoclonally propagated cultures from the BS patient were used for isolation of HLA-A2$^-$ variants by a FACStar (Becton-Dickinson) after the cells had been stained by a set of monoclonal antibodies as described above. The sorted $CD3^+$ HLA-A2$^-$ cells were used for cloning by limiting dilution. For normal donors, variants were isolated from PBMCs as described,[19] and those clones identified as recombinants were used for *HLA-DQA1* allele typing.

Chromosome analyses SCE was analyzed in PBMCs from the BS patient cultured in the presence of PHA and 5-bromo-2′-deoxyuridine, as described previously.[21] A total of 50 metaphases were analyzed. Possible chromosome changes in the HLA-A2$^-$ variants were analyzed using the G-banding method.[19]

DNA probes The probe pHLA 2a.1 derived from the 3′-untranslated region of the *HLA-A* gene[22] was provided by H. T. Orr of the University of Minnesota. The probe

detects *HLA-A* allele-specific restriction fragments in addition to several cross-hybridizing sequences.[23] The minisatellite locus probes, λMS29[24] and cMS605,[25] detect *DNF21S1* located at the distal portion of chromosome 6p (6pter-p25) and *D6S86* at chromosome 6q, respectively. cDNA from the constant region of the T-cell receptor (*TCR*)β gene[26] was provided by T. W. Mak of the Ontario Cancer Institute.

Southern blot analysis High-molecular-weight genomic DNA was extracted from wild-type and HLA-A2$^-$ variant T-cell clones for Southern blot analysis as described previously.[19]

DNA typing of the *HLA-DQA1* locus Allele typing consisted of polymerase chain reaction (PCR) amplification of *HLA-DQA1* gene exon 2 using primers GH26 and GH27[27] followed by sequence-specific oligonucleotide probe (SSOP)[27] or single-strand-conformation-polymorphism (SSCP)[28] analyses.

Estimation of spontaneous rates for HLA-A2$^-$ variants The spontaneous rates were calculated by two methods: the upper quartile method described by Armitage[29] and the mean method of Luria and Delbrück.[30] The calculations were based on the equations $q/aN - \ln(aN) = 4.09$ in the former case and $r = aN \times \ln(CaN)$ in the latter, where a is the spontaneous rate per locus per cell generation, q is the number of variants per culture in the upper quartile (75th percentile), N the number of cells per culture at the time of observation, which varied from 2×10^6 to 6×10^6 (the mean is about 3×10^6), and r the average number of variants in a total of C replicate cultures.

RESULTS

Elevated frequency of SCE Lymphocytes from the patient showed an SCE frequency of 78 ± 16 per cell (mean$\pm$SD for 50 metaphases), as typically observed among BS patients.[5]

Elevated spontaneous rates for HLA-A2$^-$ variants in cultured BS lymphocytes PBMCs separated from the blood sample of the BS patient contained too small a fraction of viable T cells, due to the time (i.e., 5 days) of shipment, to allow convincing measurement of the variant frequency. Consequently, an IL-2 dependent BS cell line (mass culture) was first established, and then secondary monoclonal cultures for fluctuation analysis were initiated 2 weeks later by limiting dilution. When the population of each secondary culture attained about 3×10^6 cells (i.e., 3 wells of a 24-well plate), frequencies of cells lacking *HLA-A2* allele expression were measured by a flow-cytometric assay.[19] To avoid repeated sampling of cells derived from the same PBMCs, which might be unduly selected for during the mass culture, each secondary culture was further tested for its pattern of *TCR* gene rearrangement, and no examples of identical patterns were found.

Representative patterns of flow-cytometric analyses of *HLA-A2* allele experssion are shown for one monoclonal T-cell culture from a normal donor (Fig. 1a) and one from the BS patient (Fig. 1b). Because of the continuous pattern for the HLA-A2 expression, the window for the enumeration of HLA-A2 defective variants was arbitrarily set as the region where 95% of cultured T cells

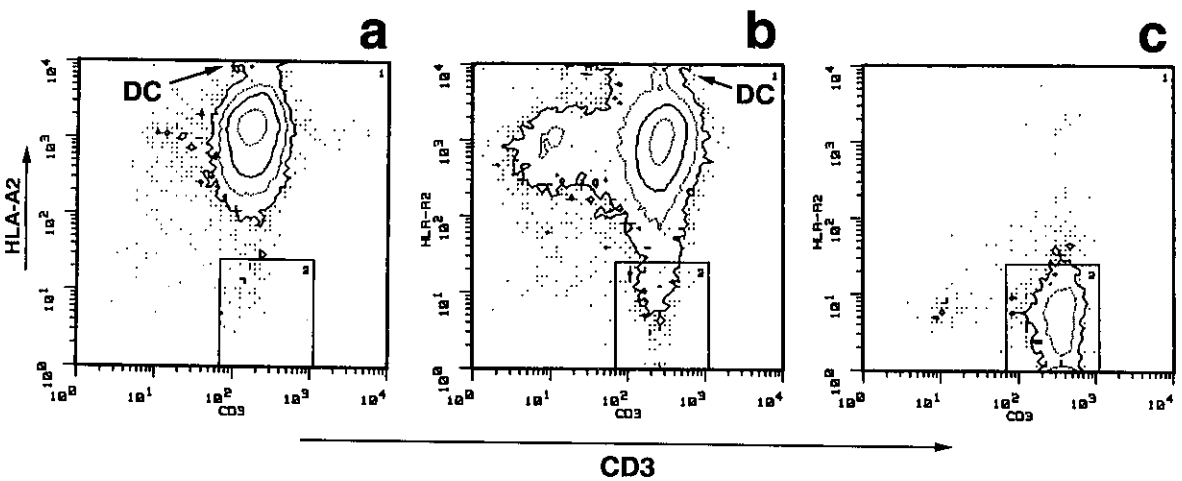

Fig. 1. Flow-cytometric patterns of monoclonally propagated T lymphocytes stained with anti-HLA-A2 and anti-CD3 antibodies. (a) A clone from normal donor 1, (b) a clone from the BS patient, (c) a clone from a donor not bearing *HLA-A2* allele. The frequencies of HLA-A2$^-$ variants are 2.4×10^{-4} in (a) and 28.7×10^{-4} in (b). The abnormal continuum is apparent for the expression of both HLA-A2 and CD3 antigens in the BS cells. Dead cells (DC) stained with propidium iodide appear at the top of the distribution.

from a laboratory volunteer without the *HLA-A2* allele are detected (Fig. 1c). About 1200 variant T cells were sorted from the mass culture of the BS patient and cultured for an additional 10 days; they showed the expected $CD3^+HLA\text{-}A2^-$ phenotype, confirming that the window for HLA-A2-deficient variants was properly set (not shown).

Fourteen secondary monoclonal populations from the BS patient showed $HLA\text{-}A2^-$ variant frequency ranging from 6.6×10^{-4} to 122×10^{-4}, with an average of 50×10^{-4} (Table I). In contrast, 23 monoclonal populations derived from PBMCs of 3 normal donors showed far lower variant frequencies, ranging from 0.4×10^{-4} to 8.5×10^{-4}, with an average of 2.1×10^{-4} (Table I).

Based on the variant frequencies of these monoclonal cultures, the spontaneous rate a for the generation of $HLA\text{-}A2^-$ variants (per locus per cell generation) was estimated using the equations described by Luria and Delbrück[30] (mean method) and by Armitage[29] (percentile method). The results of the mean method showed that $a = 50\times10^{-5}$ for BS cells and 2.8×10^{-5} for the pooled data of three normal donors (individual values were 3.6×10^{-5}, 4.1×10^{-5}, and 1.6×10^{-5} for normal donors 1, 2, and 3, respectively). The percentile method gave similar results, as shown in Table I. As was expected from the elevated average of the variant frequency, the estimated spontaneous rate was more than 10 times higher in lymphocytes derived from the BS patient than in those from normal donors.

Table I. Frequencies of $HLA\text{-}A2^-$ Variants in Monoclonal Cultures of T Lymphocytes from a BS Patient and Three Normal Donors and Estimated Spontaneous Generation Rates of the Variants

BS patient	Normal donors		
	1	2	3
Variant frequencies ($\times10^{-4}$)			
6.6, 39.6	0.4	0.6	0.4
16.6, 54.5	0.8	0.7	0.4
22.1, 57.2	1.1	1.6	0.5
28.7, 66.1	1.8	2.0	0.7
29.8, 68.5	1.8	2.1	1.1
36.3, 108	2.3	3.9	1.1
39.2, 122	2.4	8.5	2.1
	5.8		
	5.9		
average ($\times10^{-4}$)			
49.6	2.5	2.8	0.90
Spontaneous rate by the mean method (per locus per cell generation)			
50×10^{-5}	3.6×10^{-5}	4.1×10^{-5}	1.6×10^{-5}
Spontaneous rate by the percentile method (per locus per cell generation)			
58×10^{-5}	4.5×10^{-5}	4.3×10^{-5}	1.4×10^{-5}

Evidence for the somatic recombination of the variants from the BS patient About 1200 $HLA\text{-}A2^-$ cells were isolated by FACStar from the primary BS cell line after 4 weeks of culture, which showed a variant frequency of 97×10^{-4}. Through limiting dilution and *in vitro* propagation, 15 variant clones were established. Southern blot analysis for the *TCR* gene rearrangement revealed that 8 were solitary, 4 were 2 sets of duplicates and 3 were one triplicate, giving a total of 11 independent variants. In addition to these, 3 variants from one monoclonal culture and 1 from another monoclonal culture were included in the following analyses, although independent origin was not testable for the first 3 variants. The surface marker of each variant clone is shown in parentheses after the clone number in Fig. 2a.

Southern hybridization analyses using probes for the *HLA-A* gene (at 6p21.3) and two minisatellite loci (*DNF 21S1* at 6pter-6p25 and *D6S86* at 6q distal) revealed a striking feature. That is, all of the variants had lost both *HLA-A2* allele-specific *Hin*dIII fragment (5.1 kb in Fig. 2a) and 9.4 kb allele of the polymorphic *DNF21S1* locus distally located on the same chromosome 6p (Fig. 2b), whereas heterozygosity at the *D6S86* locus on the opposite arm of chromosome 6 was retained (not shown). Densitometric scanning of the autoradiographic films showed that the intensity of the 4.7 kb band (corresponding to the non-selected *HLA-Aw36* allele of the BS patient) relative to the 6.6 kb band (derived from one of the cross-hybridizing sequences[23]) was generally increased in the variants (not shown). The same is true for the 7.8 kb band of the *DNF21S1* locus compared with the 4.7 kb band, which was derived from the *DNF21S2* locus on chromosome 16[25] and served as an internal control of DNA loaded in each lane (not shown). None of the 11 $HLA\text{-}A2^+$ normal clones showed such abnormalities (not shown). G-banded metaphase chromosomes did not reveal any evidence for deletion in chromosome 6p encompassing the *HLA-A* and *DNF21S1* loci (not shown). We concluded, therefore, that all of the variants were most likely derived from somatic recombinations and not from partial deletion of chromosome 6p or from loss of one whole chromosome 6 followed by a duplication of the remaining chromosome 6.

Further, the remainder of the 1200 $HLA\text{-}A2^-$ cells, sorted from the primary BS cell line and used to establish 15 variant clones, were propagated *en masse*. Southern hybridization tests showed loss of allele-specific bands at *HLA-A* and *DNF21S1* loci but not at the *D6S86* locus as observed for the independent mutant clones, reinforcing the possibility, suggested by earlier results, that the majority of the variants are recombinants (not shown).

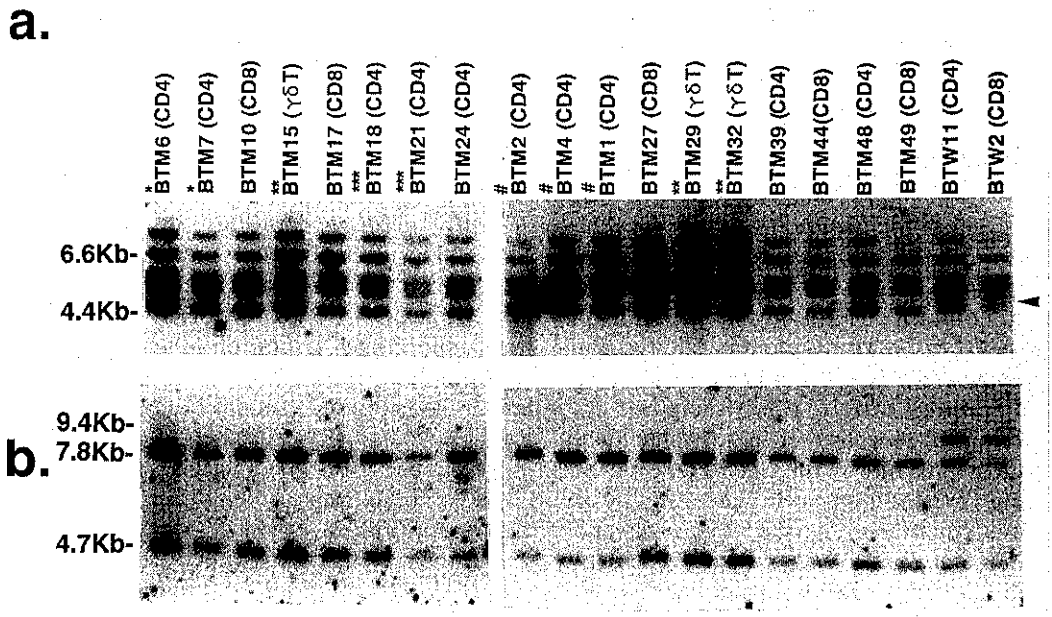

Fig. 2. Southern blot analyses of HLA-A2$^-$ variants from the BS patient. Genomic DNAs were digested with *Hin*dIII (a) or *Hin*fI (b) and were hybridized with probes pHLA2a.1 (a) or λMS29 (b). The *HLA-A2* allele-specific band (5.1 kb, shown by the arrowhead) is absent in all mutant clones but the two wild-type clones (rightmost two lanes) (a). The 9.4 kb band from one allele at the *DNF21S1* locus is also absent in all the variants (b). Note that the 9.4 and 7.8 kb bands correspond to the hypervariable *DNF21S1* locus, and the 4.7 kb band corresponds to the *DNF21S2* locus on chromosome 16. *, **, ***, represent variants with the same *TCR* rearrangement patterns, i.e., two sets of duplicates and one triplicate. # represents the three variants isolated from a monoclonal culture; the others are derived from the mass culture of the BS lymphocytes. Results for one variant from another monoclonal culture are not shown.

***HLA-DQA1* allele typing of the recombinants** Since the *HLA-A* gene selected in the present study is located at the distal portion of the HLA region, allele typing of the *HLA-DQA1* locus at the proximal part of the HLA region[31] was done using PCR-assisted allele-typing methods to test if some of the recombinations occurred within the HLA region.

PCR amplification of *HLA-DQA1* gene exon 2 followed by dot hybridization using PCR-SSOP analysis[27] or by PCR-SSCP analysis[28] revealed that the BS patient was a *HLA-DQA1*0201/*0501* heterozygote (not shown). Among the 15 HLA-A2$^-$ recombinants of the BS patient, 5 had lost heterozygosity and the remaining 10 retained it (Fig. 3). Because an independent origin for the three variants derived from one monoclonal culture cannot be verified, they were counted as one, giving a total of eight independent recombinants retaining the heterozygosity (Table II). Among the somatic recombinants of independent origin isolated from PBMCs of two normal donors in our previous study,[19] three had lost the heterozygosity and five retained it (Table II). All these results were confirmed by both PCR-SSOP and PCR-SSCP analyses.

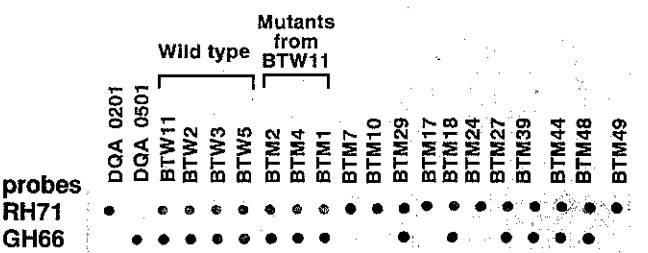

Fig. 3 *HLA-DQA1* allele typing of HLA-A2$^-$ recombinant clones from the BS patient using the polymerase chain reaction-sequence specific oligonucleotide probe method. Probes RH71 and GH66 detect the *DQA1*0201* and *DQA1*0501* allele, respectively. The leftmost two lanes represent the patterns of *HLA-DQA1*0201* and **0501* homozygotes, respectively. Four normal clones, three recombinants isolated from one monoclonal culture BTW11, and 11 independent recombinants from the mass culture are presented. One recombinant derived from another monoclonal culture is not included here but possessed both *HLA-DQA1* alleles.

Although the total number of recombinants examined is small, the distribution of recombination regions is strikingly similar for lymphocytes either from the BS

Table II. Localization of Somatic Recombinations in HLA-A Locus Recombinants

Donor	Recombination site at chromosome 6p	
	Centromere to *HLA-DQA1* (~30 Mb)	*HLA-DQA1* to *HLA-A* (~3 Mb)
Normal 1	2	2
Normal 2	1	3
BS patient	5	8
Total	8	13

patient or from normal individuals, despite the large difference in the spontaneous rate of mitotic recombination. Also the HLA region is clearly hyperrecombinogenic in somatic cells (see below).

DISCUSSION

The gene order of chromosome 6p is centromere-(*HLA-DQA1*)-(*HLA-A*)-telomere. The physical distance between the *HLA-DQA1* and *-A* loci is about 3 Mb.[31] Since the total length of chromosome 6p is estimated to be 65 Mb[32] and the HLA region (6p21.3) is approximately in the middle of 6p, we estimate that the distance between the centromere and *HLA-DQA1* locus is about 30 Mb. If somatic recombination occurs randomly, namely in proportion to physical distance, then 90% of the recombinants are expected to lose heterozygosity at the *HLA-DQA1* locus. Contrary to expectation, however, more than half of the recombinants retained the heterozygosity in both BS and non-BS cells, suggesting that the somatic recombination occurred about 10 times more frequently per unit length of DNA between the *HLA-DQA1* and *-A* loci than between the centromere and the *HLA-DQA1* locus.

The results contrast sharply with the observation that molecular length between *HLA-DP* and *-A* loci fits quite well with the map that emerged from studies of germ-line recombinational events in families, i.e., 3 Mb corresponds to about 3 cM.[33] The abnormally increased recombination frequency within the HLA region in lymphocytes suggests that either an array of structurally related genes in this region facilitates illegitimate recombination specific for somatic cells or this region is characteristically dense in the signal sequences for the mitotic recombination. Another example of non-random mitotic recombination is suggested by the distribution of Qrs; among 99 Qrs of BS lymphocytes, 17 involved chromosome 1, whereas none involved chromosome 2[34] despite the DNA content being only slightly less in chromosome 2.[32]

Because both BS and non-BS cells showed similar relative frequencies of recombination in the two regions, namely centromere to *HLA-DQA1* locus and *HLA-DQA1* to *HLA-A* loci, the defect in BS cells appears to operate in such a way as to increase the recombination frequency evenly at ordinary sites occurring in non-BS cells and not to generate unusual sites randomly (i.e., in proportion to physical length of DNA) along the chromosome 6p. Further studies to specify the recombination area in detail would provide crucial information for or against the hypothesis.

Grist *et al.*[35] collected many HLA-A mutants of lymphocytes from normal people and tested for the brief localization of somatic crossover. Interestingly, 10 out of 73 mutants derived from the recombination retained heterozygosity at the *HLA-B* locus, about 1200 kb proximal to the *HLA-A* locus. This could be a sign of increased recombination frequency in the HLA region, but the two additional polymorphic markers used did not provide enough information to confirm it. Namely, *GSTII* is far proximal (6p12.2), and *D6S10* (detected by the probe pCH6) is not mapped at the molecular level (described as 6p in Human Gene Mapping 11[31] or 6p21.3 by Morley *et al.*[36] Therefore, the present results are not incompatible with the results of Grist *et al.*[35]

Kyoizumi *et al.*[17] and Langlois *et al.*[18] reported that, in the erythrocyte mutation assay at the glycophorin A (*GPA*) locus, the flow cytogram showed a striking continuum of cells expressing varying levels of GPA between the normal peak and the mutant window. The present results add two more examples of such a continuous pattern in BS cells, *HLA-A* and *TCR* genes, as shown in Fig. 1b. Because TCR molecules need to form a complex with CD3 molecules to be expressed on the cell surface and *TCR* genes are functionally hemizygous, loss of CD3 antigen is measured here as an index for the deficiency in TCR antigen (see reference 37 for details). Langlois *et al.*[18] suggested a faulty recombination between *GPA* and related genes either by unequal SCE or chromatid exchange of homologous chromosomes, resulting in production of chimeric protein. Alternatively, such cells might also bear various base-substitution mutations resulting in varying degrees of reduced affinity of the gene products with the antibodies, or the cells might have been on their way to developing a full mutant phenotype shortly after mutations in the gene. Erythrocytes could not be used to discriminate between these alternatives because they do not contain DNA and no longer divide. When the variant lymphocytes with intermediate levels of HLA-A2 expression (*y* axis between 25 and 50 in Fig. 1b) were isolated from the primary BS cell line and grown for an additional week *in vitro*, flow-cytometric reanalysis showed that the modal peak remained essentially the same (Y. Kusunoki, in preparation). The results show the phenotype to be not transient, but stable. Further, the Southern hybridization of DNA from these

cells propagated *en masse* showed typical loss of heterozygosity at the *HLA-A* and *DNF21S1* loci, strongly suggesting that the majority of them are also recombinants (Y. Kusunoki, in preparation). Further studies are required to clarify in detail the structure of these changes.

Both *TCR* and *HLA* loci consist of many tandemly repeated genes of related structure, and such unique structures might serve as major sites for somatic recombination. In this regard, it appears relevant that frequent changes in one variable-number-of-tandem-repeat (VNTR) locus (*D1Z2*) were reported among clonal derivatives of a lymphoblastoid cell line from a BS patient but not in clonal lines from a normal donor.[38] However, none of the clonal lines, including the D1Z2 mutants, was affected at another closely but proximally mapped VNTR locus (*D1S57*). Thus, the mutations could not be attributed to large segmental events of chromosome 1p. While the possibility of intragenic homologous recombination at *D1Z2* locus cannot be formally excluded, unequal SCE was considered the most plausible mechanism by the authors. These results may imply that certain base sequences are closely involved in specific biologic changes.

The high risk of malignant diseases in BS patients clearly seems closely associated with the genomic instability. For example, the increased rate of spontaneous gene mutation[14–16] and somatic recombination (references 17, 18 and present study) can easily be envisaged to cause elevated frequencies of homozygous cells for any autosomal recessive mutations, including tumor suppressor genes.

In addition, recent studies revealed that paternal and maternal genomes are differently imprinted. Some parts of these genomes seem to counteract each other in promotion and suppression of fetal growth, and a certain disomy is suggested to predispose the fetus to tumor development.[39] In this regard, somatic recombination produces two abnormal daughter cells with partial disomy, one maternally derived and the other paternally derived. The chromosomal expression of somatic recombination, Qrs, is reported to occur at a frequency of several percent in lymphocyte cultures from 18 suspected BS patients (average of 2.4%),[34] suggesting that the somatic cells of BS patients are highly mosaic with respect to the partial disomy. These chromosomal events leading to partial disomic conditions without any loss or gain of genetic materials might also contribute to the high propensity for malignancies in BS patients.

The elevated spontaneous recombination rate in BS cells is unlikely to be attributable to the very young age of the patient (i.e., 3 years). Rather, if some age effect of the lymphocyte donor does exist, the mutation rate should be higher among the cells from older individuals. For example, the effect of exposure to environmental mutagens should increase with age, as is the case for SCE.[40] Reduction of telomeric sequence repeats is suspected to cause genomic instability and this, too, should affect more the lymphocytes from older individuals.

The lymphocyte mass culture for 2 weeks before cloning for the fluctuation test in the BS patient is unlikely to be the cause of the present results. Although *in vitro* passage of normal fibroblasts is known to result in a gradual reduction of telomere repeats that may increase genomic instability, lymphocyte clones can be continuously propagated over one year under the current culture conditions (Y. Kusunoki, unpublished observation). Thus, mass culture for 2 weeks is too short to expect induction of such an instability. That SCE frequency does not change during 9 days' *in vitro* culture of lymphocyte[41] is in accord with the above notion.

As for the possible bias for preferential survival of recombination-prone cells in the patient's blood sample during 5 days after the collection, there was no evidence for increased SCE frequency in ten other blood samples from non-BS individuals, collected and examined at the same time as the BS sample (T. Kurihara, unpublished observation). Thus, the delay in the initiation of lymphocyte culture is unlikely to have generated positive selection of phenotypically BS-like recombination-prone cells regardless of the disease status.

ACKNOWLEDGMENTS

The authors are grateful to Y. Fukuda from the Hiroshima University School of Medicine for lymphocyte typing by a standard NIH method; J. B. Cologne for his help in calculating the spontaneous-mutation rate; K. Tanabe, K. Takahashi, M. Yamaoka, T. Takahashi, and L. Dragone for their technical assistance; S. Kyoizumi for general discussions; M. Takagi and N. Saito for preparation of the manuscript; and Takeda Chemical Industries, Ltd. for the human rIL-2. This publication is based on research protocol RP11-89 and manuscript MS6-94 concerning research performed at the Radiation Effects Research Foundation (RERF), Hiroshima and Nagasaki, Japan. RERF is a private foundation funded equally by the Japanese Ministry of Health and Welfare and the US Department of Energy through the National Academy of Sciences.

(Received November 26, 1993/Accepted March 8, 1994)

REFERENCES

1) Bloom, D. Congenital telangiectatic erythema resembling lupus erythematosus in dwarfs. *Am. J. Dis. Child.*, **88**, 7546–7548 (1954).
2) German, J. Bloom's syndrome: X. The cancer proneness points to chromosome mutation as a crucial event in human neoplasia. *In* "Chromosome Mutation and Neoplasia," ed. J. German, pp. 347–357 (1983). Alan R. Liss, New York.
3) German, J. Cytogenetic evidence for crossing-over *in vitro* in human lymphoid cells. *Science*, **144**, 298–301 (1964).
4) German, J. Chromosomal breakage in a rare and probably genetically determined syndrome of man. *Science*, **148**, 506–507 (1965)
5) Chaganti, R. S. K., Schonberg, S. and German, J. A manyfold increase in sister chromatid exchanges in Bloom's syndrome lymphocytes. *Proc. Natl. Acad. Sci. USA*, **71**, 4508–4512 (1974).
6) Chan, J. Y. H., Becker, F. F., German, J. and Ray, J. H. Altered DNA ligase I activity in Bloom's syndrome cells. *Nature*, **325**, 357–359 (1987).
7) Willis, A. E. and Lindahl, T. DNA ligase I deficiency in Bloom's syndrome. *Nature*, **325**, 355–357 (1987).
8) Willis, A. E., Weksberg, R., Tomkinson, S. and Lindahl, T. Structural alterations of DNA ligase I in Bloom syndrome. *Proc. Natl. Acad. Sci. USA*, **84**, 8016–8020 (1987).
9) Heartlein, M. W., Tsuji, H. and Latt, S. A. 5-Bromodeoxyuridine-dependent increase in sister-chromatid exchange formation in Bloom's syndrome is associated with reduction in topoisomerase II activity. *Exp. Cell Res.*, **169**, 245–254 (1987).
10) Seal, G., Brech, K., Karp, S. J., Cool, B. L. and Sirover, M. A. Immunological lesions in human uracil DNA glycosylase: association with Bloom syndrome. *Proc. Natl. Acad. Sci. USA*, **85**, 2339–2343 (1988).
11) Shiraishi, Y., Taguchi, T., Ozawa, M. and Bamezai, R. Different mutations responsible for the elevated sister-chromatid exchange frequencies in Bloom syndrome and X-irradiated B-lymphoblastoid cell lines originating from acute leukemia. *Mutat. Res.*, **211**, 273–278 (1989).
12) Nicotera, T. M., Notaro, J., Notaro, S., Schumer, J. and Sandberg, A. A. Elevated superoxide dismutase in Bloom's syndrome: a genetic condition of oxidative stress. *Cancer Res.*, **49**, 5239–5243 (1989).
13) McDaniel, L. and Schultz, R. A. Elevated sister chromatid exchange phenotype of Bloom syndrome cells is complemented by human chromosome 15. *Proc. Natl. Acad. Sci. USA*, **89**, 7968–7972 (1992).
14) Gupta, R. S. and Goldstein, S. Diphtheria toxin resistance in human fibroblast cell strains from normal and cancer-prone individuals. *Mutat. Res.*, **73**, 331–338 (1980).
15) Warren, S. T., Schultz, R. A., Change, C. C., Wade, M. H. and Trosko, J. E. Elevated spontaneous mutation rate in Bloom's syndrome fibroblasts. *Proc. Natl. Acad. Sci. USA*, **78**, 3133–3137 (1981).
16) Vijayalaxmi, Evans, H. J., Ray, J. H. and German, J. Bloom's syndrome: evidence for an increased mutation frequency *in vivo*. *Science*, **221**, 851–853 (1983).
17) Kyoizumi, S., Nakamura, N., Takebe, H., Tatsumi, K., German, J. and Akiyama, M. Frequency of variant erythrocytes at the glycophorin A locus in two Bloom's syndrome patients. *Mutat. Res.*, **214**, 215–222 (1989).
18) Langlois, R. D., Bigbee, W. L., Jensen, R. H. and German, J. Evidence for increased *in vivo* mutation and somatic recombination in Bloom's syndrome. *Proc. Natl. Acad. Sci. USA*, **86**, 670–674 (1989).
19) Kushiro, J., Hirai, Y., Kusunoki, Y., Kyoizumi, S., Kodama, Y., Wakisaka, A., Jeffreys, A., Cologne, J. B., Dohi, K., Nakamura, N. and Akiyama, M. Development of a flow-cytometric HLA-A locus mutation assay for human peripheral blood lymphocytes. *Mutat. Res.*, **272**, 17–29 (1991).
20) Kusunoki, Y., Hirai, Y., Kyoizumi, S. and Akiyama, M. Evidence for *in vivo* clonal proliferation of unique population of blood $CD4^-/CD8^-$ T cells bearing T-cell receptor α and β chains in two normal men. *Blood*, **79**, 2965–2972 (1992).
21) Kurihara, T., Tatsumi, K., Takahashi, H. and Inoue, M. Sister-chromatid exchanges induced by ultraviolet light in Bloom's syndrome fibroblasts. *Mutat. Res.*, **183**, 197–202 (1987).
22) Koller, B. H., Sidwell, B., DeMars, R. and Orr, H. Isolation of HLA locus-specific DNA probes from the 3′-untranslated region. *Proc. Natl. Acad. Sci. USA*, **81**, 5175–5178 (1984).
23) Koller, B. H., Geraghty, D., Orr, H. T., Shimizu, Y. and DeMars, R. Organization of the human class I major histocompatibility complex genes. *Immunol. Res.*, **6**, 1–10 (1987).
24) Wong, Z., Royle, N. J. and Jeffreys, A. J. A novel human DNA polymorphism resulting from transfer of DNA from chromosome 6 to chromosome 16. *Genomics*, **7**, 222–234 (1990).
25) Armour, J. A. L, Povey, S., Jeremiah, S. and Jeffreys, A. J. Systematic cloning of human minisatellites from ordered array chromid libraries. *Genomics*, **8**, 501–512 (1990).
26) Yanagi, Y., Yoshikai, Y., Laggett, K., Clark, S. P., Aleksander, I. and Mak, T. W. Human T cell-specific cDNA clone encodes a protein having extensive homology to immunoglobulin chains. *Nature*, **308**, 145–149 (1984).
27) Saiki, R. K., Walsh, P. S., Levenson, C. H. and Erlich, H. A. Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes. *Proc. Natl. Acad. Sci. USA*, **86**, 6230–6234 (1989).
28) Hayashi, T., Seyama, T., Ito, T., Kusunoki, Y., Hirai, Y., Nakamura, N. and Akiyama, M. A simple and rapid method for HLA-DQA1 genotyping by polymerase chain reaction-single strand conformation polymorphism and

restriction enzyme cleavage analysis. *Electrophoresis,* **13,** 877–879 (1992).
29) Armitage, P. J. The statistical theory of bacterial populations subject to mutation. *J. R. Stat. Soc. B,* **14,** 1–40 (1952).
30) Luria, S. E. and Delbrück, M. Mutation of bacteria from virus sensitivity to virus resistance. *Genetics,* **28,** 491–511 (1943).
31) Ziegler, A., Field, L. L. and Sakaguchi, A. Y. Report of the committee on the genetic constitution of chromosome 6. *Cytogenet. Cell Genet.,* **58,** 295–336 (1991).
32) Morton, N. E. Gene maps and location data bases. *Ann. Hum. Genet.,* **55,** 235–241 (1991).
33) Olaisen, B., Sakaguchi, A. Y. and Naylor, S. L. Report of the committee on the genetic constitution of chromosomes 5 and 6. *Cytogenet. Cell Genet.,* **46,** 147–169 (1987).
34) German, J., Crippa, L. P. and Bloom, D. Bloom's syndrome: III. Analysis of the chromosome aberration characteristic of this disorder. *Chromosoma,* **48,** 361–366 (1974).
35) Grist, S. A., McCarron, M., Kutlaca, A., Turner, D. R. and Morley, A A. *In vivo* human somatic mutation: frequency and spectrum with age. *Mutat. Res.,* **266,** 189–196 (1992).
36) Morley, A. A., Grist, S. A., Turner, D. R., Kutlaca, A. and Bennett, G. Molecular nature of *in vivo* mutations in human cells at the autosomal HLA-A locus. *Cancer Res.,* **50,** 4584–4587 (1990).
37) Kyoizumi, S., Akiyama, M., Hirai, Y., Kusunoki, Y., Tanabe, K. and Umeki, S. Spontaneous loss and alteration of antigen receptor expression in mature $CD4^+$ T cells. *J. Exp. Med.,* **171,** 1981–1999 (1990).
38) Groden, J. and German, J. Bloom's syndrome: XVIII. Hypermutability at a tandem-repeat locus. *Hum. Genet.,* **90,** 360–367 (1992).
39) Little, M., van Heyningen, V. and Hastie, N. Dads and disomy and disease. *Nature,* **351,** 609–610 (1991).
40) Wulf, H. C., Kousgaard, N. and Niebuhr, E. Sister-chromatid exchange in childhood in relation to age and sex. *Mutat. Res.,* **174,** 309–312 (1986).
41) Giulotto, E., Mottura, A., Giorgi, R., deCarli, L. and Nuzzo, F. Frequencies of sister-chromatic exchanges in relation to cell kinetics in lymphocyte cultures. *Mutat. Res.,* **70,** 343–350 (1980).