CLONING OF PHENOTYPICALLY DIFFERENT HUMAN LYMPHOCYTES ORIGINATING FROM A SINGLE STEM CELL

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The analysis of lymphocytes derived from a single stem cell at various stages of development provides a fascinating model for the study of their differentiation. This is possible in mice by means of transplanting a cell population containing stem cells that carry some specific markers, such as chromosome aberrations, alloantigens, or exogenous genes (1–3). Using these methods, it has been suggested that a population of stem cells is committed to differentiate into a T cell lineage. IL-3-dependent, bone marrow-derived mouse cell lines that can differentiate into either T cells or B cells also have been reported (4, 5).

In humans, myeloproliferative disorders, such as chronic myelogenous leukemia, polycythemia vera, and primary thrombocytosis, may originate from single cells in the pluripotent hematopoietic stem cell pool (6–8). However, useful models or cell lines for the study of human lymphocyte differentiation have not yet been established.

The identification of in vivo derived cellular markers in humans would preclude the use of mouse procedures, such as bone marrow transplantation, for the study of lymphocyte differentiation. In this respect, it should be noted that it now is possible to clone selectively rare (on the order of 10^{-5}–10^{-6}) T cells lacking enzyme activity for hypoxanthine guanine phosphoribosyltransferase (HPRT),[1] a member of the purine salvage pathway, from the human peripheral blood (9, 10). The cells deficient in HPRT activity can be selected, taking advantage of their ability to grow in the presence of a purine analogue, 6-thioguanine (TG). These TG-resistant (TG^r) T cells are the result of gene mutation at the *hprt* locus and have altered patterns of *hprt* DNA that differ among various mutant clones (11–14). Thus, *hprt* gene alterations can be used as in vivo cellular markers.

We have been studying somatic cell mutations in cloned TG^r T cells in atomic bomb survivors from Hiroshima (15, 16). In this population, a high proportion of

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[1] *Abbreviations used in this paper:* CE, cloning efficiencies; HPRT, hypoxanthine guanine phosphoribosyltransferase; TG, 6-thioguanine; TG^r, TG resistant.

peripheral T cells have radiation-induced chromosome aberrations (17). Since these aberrations have been randomly induced, aberration patterns differ from one cell to another. Thus, chromosome aberrations also can be used as in vivo cell markers in atomic bomb survivors.

Maturing lymphocytes with different functions are known to express several different cell surface markers (18, 19) and to rearrange their antigen receptor genes to produce diversity (20, 21). The identification of human T, NK, and B cells originating from a single stem cell will be presented here by using *hprt* gene alterations and chromosome aberrations as markers for cell clonality and surface phenotypes, and gene rearrangements of TCRs and Igs as differentiation markers.

Materials and Methods

Preparation of PBMC. PBMC were obtained from a male atomic bomb survivor in Hiroshima, whose DS86 radiation dose (shielded kerma) estimate from atomic bomb exposure was 1.99 Gy (22). He was 59 yr old at the time of examination (his age at the time of exposure to atomic bomb radiation was 17 yr) and had no apparent physical disorders.

Venous peripheral blood was defibrinated with glass beads and PBMC were recovered by Ficoll-Hypaque density centrifugation. PBMC were washed twice with Earl's balanced salt solution (Nissui, Tokyo, Japan) before use.

Cloning of IL-2-dependent Normal and TG^r Lymphocytes. Full details of our cloning method have been described previously (15). The basic methodology was originally reported by Albertini et al. (9). In brief, fresh PMBC, mixed with PHA (Difco Laboratories, Detroit, MI), IL-2, and feeder cells, were inoculated into the wells of microtiter plates (Costar, Cambridge, MA). On the average, 5×10^3 cells/well were inoculated into wells with TG for the cloning to TG^r T cells. Normal T cells were cloned in the wells that received PBMC at a concentration of one cell per well. After 15 d of culture, growing colonies in each well were identified by means of an inverted microscope. They then were isolated and subcultured for further analysis.

Cloning efficiencies (CE) were calculated from the proportion of colony-negative wells, assuming a Poisson distribution of the cells having the ability to form colonies. The frequency of TG^r mutant lymphocytes was obtained by dividing the CE of TG-selected lymphocytes by the CE of normal cells.

Cloning of Normal and TG^r B Cells. To isolate normal and TG^r mutant B cells, PBMC were infected with EBV from a culture supernatant of the B 95-8 marmoset cell line. After 2 h of incubation at 37°C, the culture supernatant was replaced with fresh RPMI 1640 medium supplemented with 10% FCS and 1% L-glutamine. An average of 10^5 or 10^3 infected cells was inoculated into the wells of the microtiter plates either with or without TG. After 2–4 wk of culture, changing half of the medium of each well once a week, colonies were isolated and subcultured.

The CE and the frequency of TG^r B cells were calculated in the same way in which those of the IL-2-dependent TG^r lymphocytes were calculated.

Chromosome Analysis of the Lymphocyte Colonies. Lymphocyte colonies were examined for karyotype analysis from 3 wk to 2 mo after initiation of the cultures. Standard cytogenetic procedures were used for preparing G-banded chromosomes (23). From 1 to 10 metaphases per colony were examined.

Extraction of Genomic DNA. Lymphocyte colonies were increased in number to $>3 \times 10^6$ and then were frozen at −70°C in preparation for DNA extraction. The frozen cells were thawed and suspended in 0.3 ml of PBS, after which 1–5 ml of digestion buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA, 0.5% SDS), containing 100 μg/ml RNase A (Sigma Chemical Co., St. Louis, MO), was added. After 1 h of incubation at 37°C, 1–5 ml of digestion buffer containing 400 μg/ml of proteinase K (Boehringer Mannheim, Mannheim, FRG) was added and the mixture was incubated at 37°C for 4 h. The solution was phenol/chloroform (2:1) extracted, dialized against $T_{10}E_1$ (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and then stored at 4°C.

Southern Blot Hybridization. For the Southern blots, 5 μg of DNA was digested to completion with restriction enzymes, fractionated on a 0.7% agarose gel in Boyer's buffer (50 mM Tris-HCl, pH 8.0, 20 mM Na-acetate, 2 mM EDTA, 10 mM NaCl), and transferred to nitrocellulose filters (Schleicher & Schuell, Dassel, FRG), using 20× SSC. Prehybridization was conducted for 3 h at 65°C in 50 mM Tris-HCl, pH 7.8, 10 mM EDTA, 1 M NaCl, 10× Denhart's solution, 0.1% SDS. Hybridization was carried out for 12–16 h at 65°C in 2 ml of the same solution used in prehybridization containing 100 μg/ml denatured salmon sperm DNA. The probes were labeled with [^{32}P]dCTP (Amersham International, Amersham, UK) to a sp act of 2×10^7–7×10^7 cpm/μg using a random primer method (Amersham International). 15–30 ng of probes was added to each filter. Washing of hybridized filters was performed at 65°C in 0.5 × SSC, 0.1% SDS for *hprt*, and in 0.1 × SSC, 0.1% SDS for the other probes. Autoradiography was conducted at −80°C with intensifying screens (DuPont Co., Wilmington, DE).

Probes. Alterations of the *hprt* gene were examined using a 941-bp, full-length cDNA gene probe (pHPT30 cleaved with Pst I) (24), kindly provided by Dr. C. T. Caskey, Howard Hughes Medical Institute, Baylor College of Medicine, Houston, TX. Gene rearrangements of the TCR β and γ chains were studied with a 770-bp cDNA constant region gene probe (25), kindly provided by Dr. T. W. Mak, Ontario Cancer Institute, Toronto, Ontario, Canada, and a 700-bp genomic probe of Jγ, courtesy of Dr. T. H. Rabbitts, Medical Research Council, UK, respectively. Ig H chain gene rearrangements were analyzed with a 4.5-kb Eco RI–Hind III fragment from a germ-line Cμ gene clone (26), the generous gift of Dr. T. Honjo, Kyoto University, Kyoto, Japan.

Analysis of Surface Phenotypes. Surface phenotypes of the cloned lymphocytes were analyzed using a FACS analyzer (Becton Dickinson & Co., Mountain View, CA). mAbs used were Leu-4 (anti-CD3), Leu-3 (anti-CD4), Leu-2 (anti-CD8), Leu-5 (anti-CD2), Leu-11 (anti-CD16), Leu-7, anti-κ, and anti-λ, all obtained from Becton Dickinson & Co., and NKH-1 from Coulter Electronics Inc. (Hialeah, FL).

Cytotoxicity Assay. Cytotoxic activity of the IL-2-dependent colonies against K562 cells was measured using the ^{51}Cr-releasing method. Target cells were labeled with 100 μl of ^{51}Cr (New England Nuclear, Boston, MA) for 1 h at 37°C and were washed extensively before use. Effector cells were plated with 10^3 target cells in 200 μl in microtiter plates with U-bottomed wells (Costar). The E/T ratio ranged from 5:1 to 20:1. After 3 h of incubation at 37°C, the plates were centrifuged and the supernatant was assayed for the release of ^{51}Cr from the target cells. Two or three replicates per experimental group were used, and the percentage of specific lysis was calculated by the formula: percent specific lysis = (cpm in experimental wells − cpm in wells with target cells alone)/cpm incorporated in target cells.

Results

Cloning of IL-2-dependent Normal and TG^r Lymphocytes. After 15 d of culture, growing colonies were observed in 19 of 96 nonselected wells and in 21 of 96 TG-selected wells. Cloning efficiencies, calculated from the proportion of colony-negative wells, were 0.22 and 0.50×10^{-4} for normal and TG^r cells, respectively, yielding a TG^r mutant frequency of 2.3×10^{-4}. This mutant frequency is 20–100 times higher than the frequencies observed in 30 other atomic bomb survivors (16) or the frequencies reported for normal individuals (9, 10, 15). PBMC were obtained again 5 mo later and a similar frequency of TG^r cells was observed (2.6×10^{-4}). The IL-2-dependent colonies obtained from these two experiments were used for further analysis.

Cloning of TG^r B Cells. Growing colonies were observed 2–4 wk after initiation of the cultures in the wells that had been inoculated with EBV-infected cells. 38 of 96 nonselected wells and 73 of 240 TG-selected wells showed colony growth, yielding a TG^r B cell frequency of 7.2×10^{-3}. Colonies were isolated and subcultured for further analysis.

Chromosome Analysis of the Lymphocyte Colonies. Chromosome analysis was performed on 32 TG^r IL-2-dependent and 30 EBV-transformed TG^r lymphocyte colonies. As shown in Fig. 1, all the TG^r colonies showed the same abberration, a terminal deletion of the long arm of chromosome 20. On the other hand, 7 of 14 IL-2-dependent and 11 of 24 EBV-transformed normal lymphocyte colonies showed various different types of radiation-induced aberrations (mostly reciprocal translocations). These results indicate that all the TG^r lymphocytes derived from a single cell.

Alterations of the hprt Gene of the TG^r Colonies. The single-cell origin of the TG^r lymphocytes also was demonstrated by means of Southern blotting analysis, using *hprt* cDNA as a probe. All of the TG^r lymphocyte colonies showed the same alterations (appearance of novel bands) when DNA was cut with the restriction enzyme Pvu II or Bgl II (Fig. 2). No alterations were observed when the DNA was cut with Bam HI, Eco RI, Hind III, Pst I, or Msp I (data not shown in Fig. 2). Southern blotting analysis of *hprt* genes of spontaneously occurring in vivo derived TG^r T cells has shown that those mutant cells vary in their types of alterations (11–14) (i.e., no *hrpt* gene "hot spots" have been observed). Thus, the common *hprt* alteration observed in this study was evidence for the single-cell origin of the TG^r lymphocytes. Because both IL-2-dependent and EBV-transformed lymphocytes were found to be derived from a single cell, the original cell was a stem cell that differentiated into both T and B cells. To further characterize the original stem cell, we investigated rearrangements of TCR and Ig genes and surface phenotypes of the TG^r lymphocyte colonies.

Analysis of TCR and Ig Gene Rearrangements. Fig. 3 shows the gene rearrangements of the TCR β and γ chains of the IL-2-dependent colonies. All of the normal and TG^r colonies showed multiple rearrangement patterns, except for $T30^r$ and $T4^r$, which were of the NK cell phenotype (see Table I and next section). Multiple rearrangements of the Ig H chain genes of the EBV-transformed TG^r colonies were also noted (Fig. 4).

Surface Phenotypes. The cell surface phenotypes of lymphocyte colonies were studied using immunofluorescence staining. As shown in Table I, the IL-2-dependent TG^r colonies were of various phenotypes, including classic T cells ($CD3^+4^+8^-$ or $CD3^+4^-8^+$) and a "double-negative" T cell ($CD3^+4^-8^-$). Some of the mutant colonies with various phenotypes (such as $CD3^-16^+$) exhibited cytotoxic activity against the NK-sensitive cell line K562 (cytotoxic activity was 50–80% at an E/T ratio of 20:1). Surface phenotype analysis of normal IL-2-dependent colonies showed that 12 of 13 colonies were positive for CD3 classic T cell phenotypes (nine were $CD4^+8^-$, and three were $CD4^-8^+$). Only one normal colony lacked the CD3 antigen ($CD3^-4^-8^+$). However, as shown in Table I, 6 of 27 TG^r colonies lacked CD3. Although not listed in the table, the other six TG^r colonies were a mixture of $CD3^+$ and $CD3^-$ cells (all these colonies showed cytotoxic activity against K562 cells). Therefore, the proportion of $CD3^-$ cells was higher in TG^r colonies than in the normal colonies. The ratio of $CD4^+$ to $CD8^+$ classic T cells was similar in TG^r and normal colonies, results that were similar to those reported previously for different donors (15).

L chain isotypes of the surface Ig of EBV-transformed lymphocyte colonies were analyzed. Analysis of 13 TG^r colonies showed that nine had κ and four had λ isotypes. Analysis of 12 normal colonies indicated that nine had κ and three had λ isotypes.

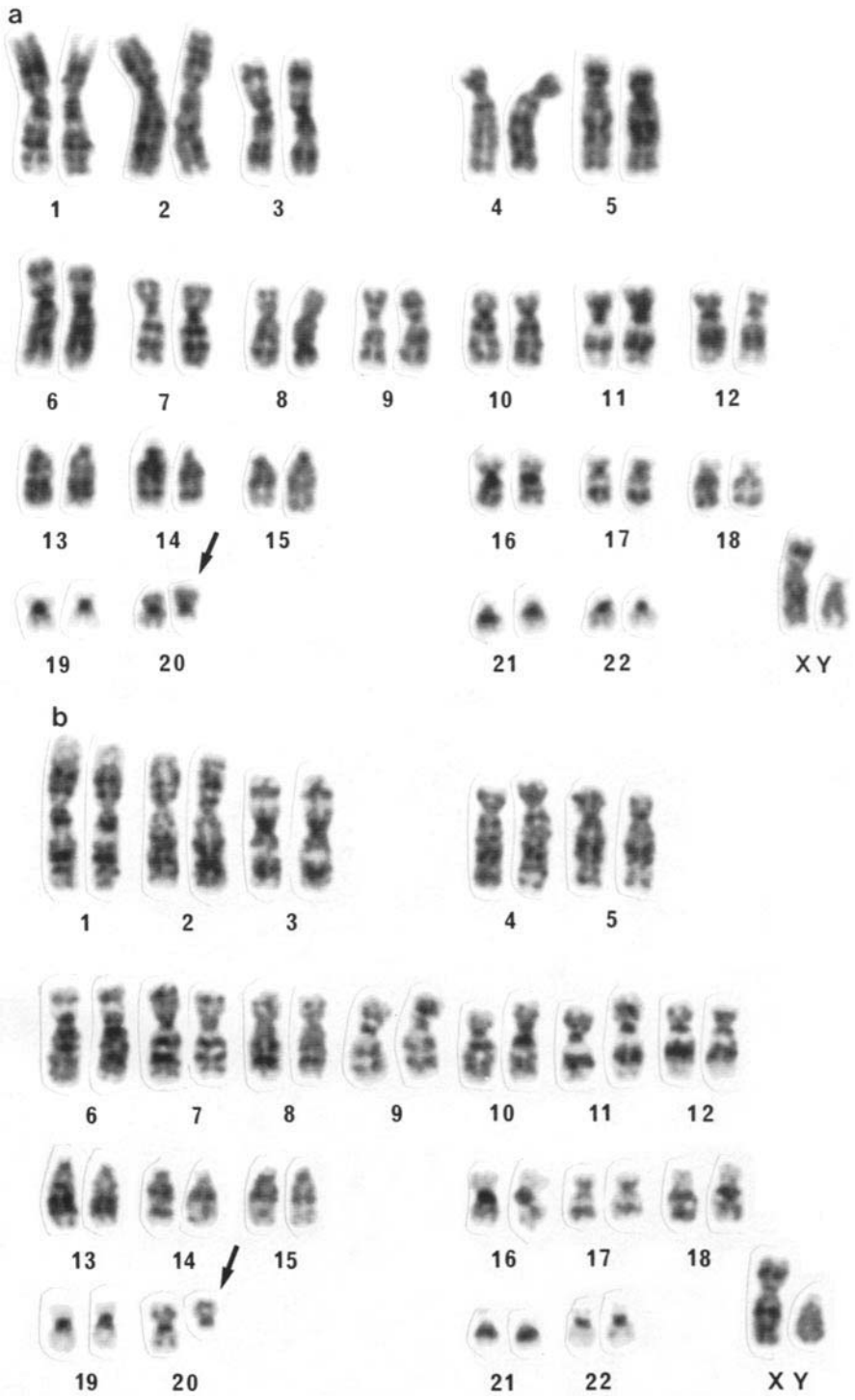

FIGURE 1. Representative karyotypes with G banding of cells from the TG^r lymphocyte colonies, T23^r (*a*) and B13^r (*b*). In both colonies, G banding revealed a terminal deletion (*arrow*) of the long arm of chromosome 20 with the breakpoint at q 11.2. An identical karyotype was observed in the other TG^r colonies.

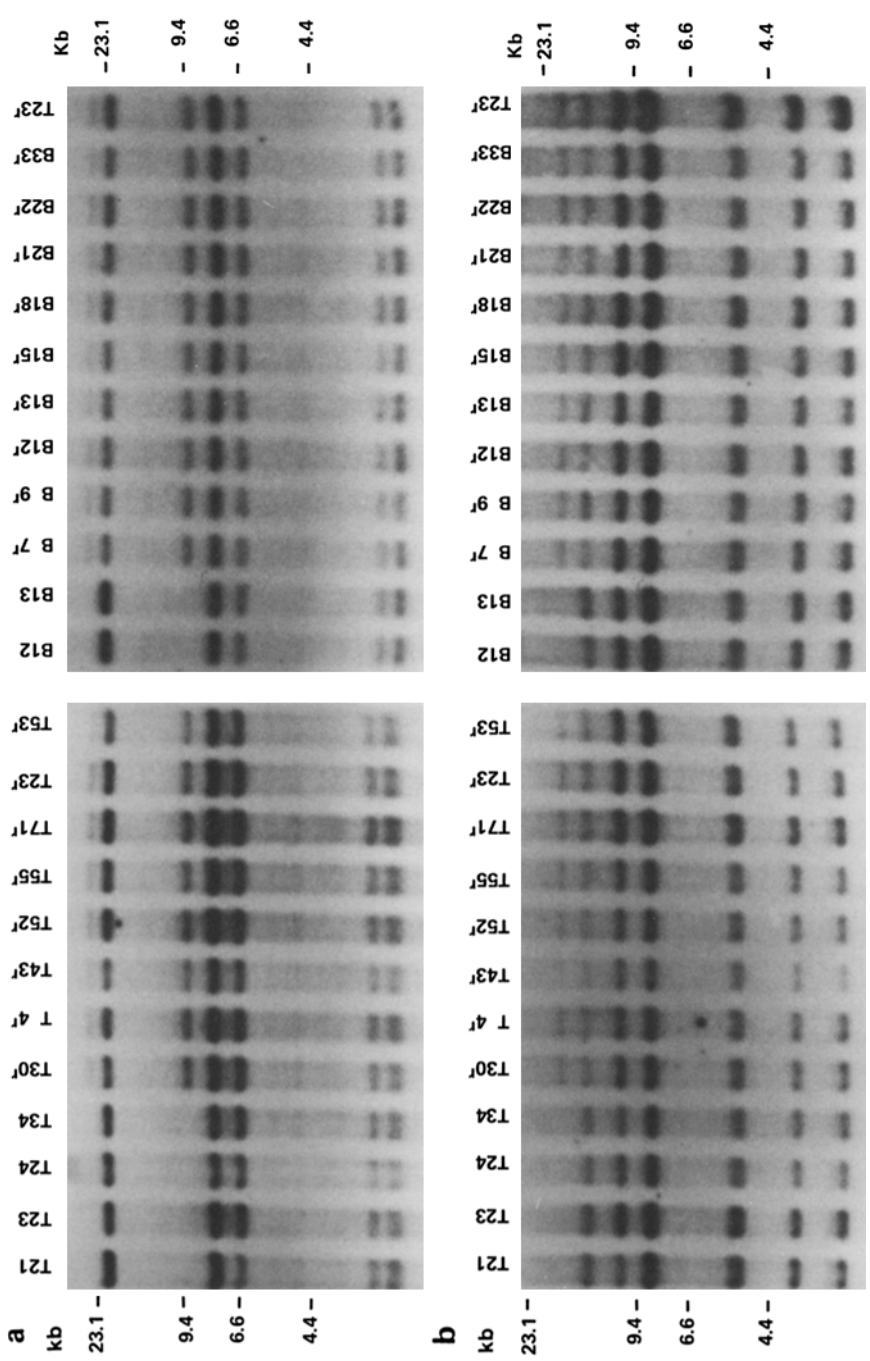

FIGURE 2. Southern blot patterns of four normal (T21, T23, T24, T34) and eight TG^r (T30^r, T4^r, T43^r, T52^r, T55^r, T71^r, T23^r, T53^r) IL-2-dependent lymphocytes, and two normal (B12, B13) and nine TG^r (B7^r, B9^r, B12^r, B13^r, B15^r, B18^r, B21^r, B22^r, B33^r) EBV-transformed B cell colonies. One TG^r T cell colony, T23^r, was analyzed again on the filters where B cell colonies were analyzed. (*a*) *Pvu* II–digested genomic DNA was hybridized with a labeled full-length *hprt* probe. The appearance of novel bands of the same sizes (27 and 9.2 kb) was observed in all the TG^r colonies. (*b*) Bgl II–digested genomic DNA was hybridized with a labeled full-length *hprt* probe. The appearance of a novel band of the same size (18.5 kb) was observed in all the TG^r colonies.

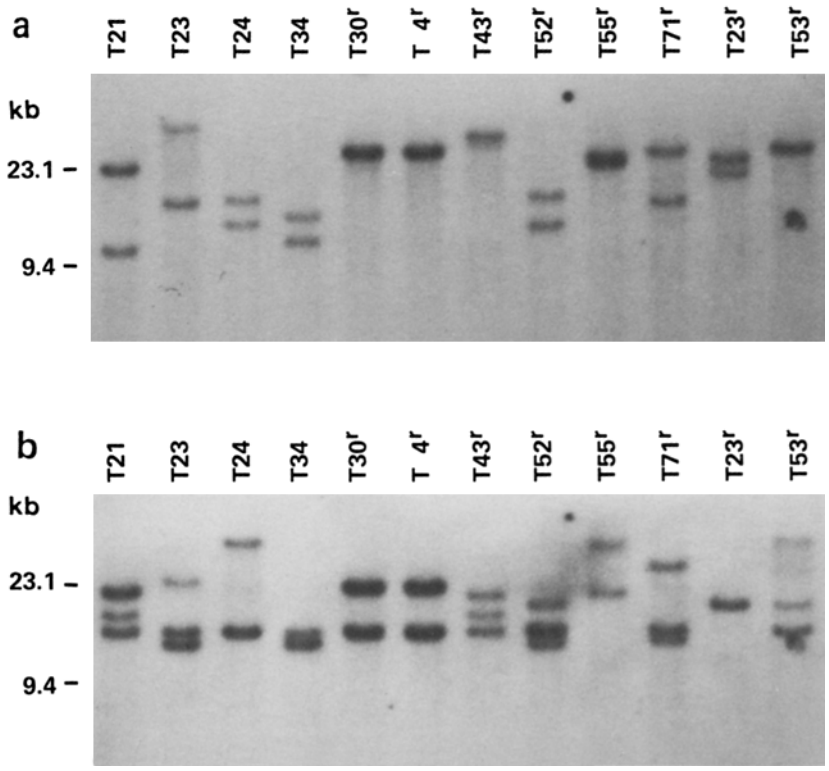

FIGURE 3. Southern blot patterns of four normal (T21, T23, T24, T34) and eight TG^r ($T30^r$, $T4^r$, $T43^r$, $T52^r$, $T55^r$, $T71^r$, $T23^r$, $T53^r$) IL-2-dependent lymphocyte colonies. (*a*) Bam HI-digested genomic DNA was hybridized with a labeled TCR β chain constant region probe. (*b*) The same filter was rehybridized with a labeled J region genomic fragment of the TCR γ chain.

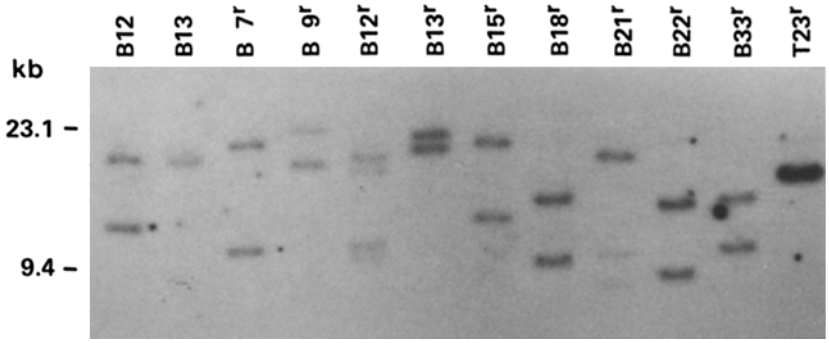

FIGURE 4. Southern blot patterns of two normal (B12, B13) and nine TG^r ($B6^7$, $B9^r$, $B12^r$, $B13^r$, $B15^r$, $B18^r$, $B21^r$, $B22^r$, $B33^r$) EBV-transformed B cell colonies and of one TG^r T cell colony ($T23^r$). Eco RI-digested genomic DNA was hybridized with a labeled IgH chain J region genomic probe.

TABLE I

Characteristics of Mutant Colonies Propagated in IL-2

Mutant colonies	Surface phenotype							Cytotoxicity against K 562	Number of mutant colonies with similar characteristics
	CD3	CD4	CD8	CD2	CD16	NKH1	Leu-7		
T 30^r	−	−	−	+	+	+	−	+	3
T 4^r	−	−	+	+	+	+	−	+	2
T 43^r	−	+	−	+	−	−	−	−	1
T 52^r	+	−	−	+	−	−	−	+	1
T 55^r	+	+	−	+	−	−	+	+	3
T 71^r	+	+	−	+	−	−	−	+	2
T 23^r	+	+	−	+	−	−	−	−	9
T 53^r	+	−	+	+	−	−	−	−	3

A mixture of CD3$^+$ and CD3$^-$ cells was observed in six TG^r colonies that were not included in this table.

Thus, it has been shown that the stem cell, origin of the TG^r lymphocytes, differentiated into several subsets of T cell lineage, into NK cells, and also into B cells.

Discussion

Different cloned human HPRT$^-$ lymphocytes, belonging to several subsets of T cells, NK cells, and B cells, were shown to originate from a single stem cell by demonstrating that they shared two cellular markers, chromosome aberrations, and *hprt* gene alterations. T and B cell colonies showed multiple gene rearrangement patterns of the TCR β and γ chains and Ig H chains, respectively. This indicated the diversity of their antigen specificities. Of course, there is a possibility that the original stem cell could also differentiate into cells of myeloid or erythroid lineage. However, it is hard to test this possibility because of the lack of efficient culture methods for these two cell lineages.

To estimate when the original stem cell initiated differentiation into mature lymphocytes was possible from chromosome analysis of the cloned TG^r lymphocytes. The chromosome aberrations observed in TG^r lymphocytes indicated that the stem cell began to differentiate subsequent to atomic bomb radiation exposure because the frequency of bone marrow cells and PBL bearing chromosome aberrations is very low (0–1%) in healthy persons not exposed to radiation. However, in atomic bomb survivors, the frequency is much higher (>10% in persons exposed to the bomb within 1,000 m) (17, 27, 28). The chromosome aberration frequency of PHA-stimulated PBL from the person in our study was 21% (17), and it is considered that most of these aberrations were atomic bomb radiation induced. Thus, the chromosome aberrations in the TG^r colonies may also have been induced by atomic bomb radiation, as has been substantially supported. If the TG^r cell population had existed at the time of atomic bomb exposure, various other types of chromosome aberrations should have been observed in different subsets of the TG^r colonies because of the high level of atomic bomb induction of different types of such aberrations. However, all of the TG^r colonies (32 IL-2-dependent and 30 B cell colonies) that were examined showed only a common partial deletion of the long arm of chromosome 20. On the other hand, analysis of normal colonies from the same person showed that ~50% of both

T and B cell colonies (7 of 14 IL-2-dependent and 9 of 21 B cell colonies) had various types of aberrations, indicating that a high number of aberrations was induced by radiation exposure. Another study has shown that 9 of 20 normal and 22 of 49 TG^r T cell colonies obtained from nine other atomic bomb survivors had various different types of radiation-induced aberrations (Kodama, Y., M. Hakoda, H. Shimba, A. A. Awa, and M. Akiyama, manuscript submitted for publication). This indicates that chromosome aberrations could have been equally induced in both TG^r cells and normal cells if they both had been present at the time of atomic bomb exposure. These observations strongly suggest that the TG^r T, NK, and B cells emerged after atomic bomb exposure. Since the person studied was 17 yr old at exposure, the observations in this individual suggest that there are common stem cells for at least T, NK, and B cells as late in life as 17 yr of age.

Differences in the mechanisms with which stem cells differentiate into various cell types might explain why the B cell frequency of TG^r cells was ~35 times higher than that of the T cells in this subject. The thymus is known to be the central organ for differentiation of cells of the T cell lineage (29), and it is well known that involution occurs after adolescence (30). Commencement of thymic involution at the age of 17 might have been expected to disturb mutant stem cell differentiation into cells of the T cell lineage. A higher frequency of $CD3^-$ IL-2-dependent cells with NK cell activity in TG^r lymphocyte population than in a normal population might also be attributable to age-dependent thymic involution. The high levels of NK activity observed in nude mice and neonatally thymectomized mice are consistent with the hypothesis that NK cells accumulate when absence of the thymus prevents differentiation of stem cells into mature T cells (31).

Another possibility to be considered is that the differences in frequencies between TG^r T cells and B cells and $CD3^-$ cells in comparison with TG^r and normal IL-2-dependent cells are due to HPRT enzyme deficiency in the TG^r cells. This seems unlikely, however, since the cells of persons with Lesch-Nyhan syndrome are totally deficient in HPRT activity, yet these persons have a normal number of peripheral blood T cells (32). Lack of difference of the composition of IL-2-dependent lymphocyte subsets between TG^r and normal lymphocytes has been reported in Lesch-Nyhan heterozygotes, a portion of whose lymphocytes lack HPRT activity (33). We have previously reported on the surface phenotypes of spontaneously occurring TG^r cells in normal individuals (15). No difference was observed in the distribution of lymphocyte subsets in relation to TG resistance. Thus, it seems unlikely that *hprt* enzyme deficiency played a significant role in suppression of differentiation of cells of the T cell lineage.

Further investigation of the types of stem cell mutations, which have occurred at various stages of differentiation, may provide additional information on the process of lymphocyte differentiation. If there is the expected radiation-induced decrease in the number of hematopoietic stem cells in atomic bomb survivors, the number of residual or induced TG^r stem cells should be increased. This should make it easier to find additional stem cell mutations in the survivors than in the normal population. Furthermore, since a large number of chromosome aberrations with random distribution patterns were induced in the cells of this particular population, we are able to use such aberrations as well as *hprt* gene alterations as cell markers for determining clonality. We believe that our studies provide a novel way to identify human

stem cells in the lymphocyte lineage and to investigate the differentiation of human lymphocytes by means of an in vivo somatic mutation assay.

Summary

By using hypoxanthine guanine phosphoribosyltransferase (*hprt*) gene alterations and chromosome aberrations as in vivo cellular markers, human T, NK, and B cells originating from a single stem cell have been successfully cloned from the peripheral blood of an atomic bomb survivor from Hiroshima. These mutant lymphocytes were selectively cloned, taking advantage of their resistance to a purine analogue, 6-thioguanine. The cloned lymphocytes possessed the same *hprt* gene alterations and the same chromosome aberration ($20q^-$), but exhibited different surface or functional phenotypes and different rearrangements of TCR or Ig genes. The chromosome aberration patterns strongly suggested that the original stem cell initiated differentiation into each cell type after exposure to atomic bomb radiation. Since the person studied here was exposed to the bomb at 17 yr age, the results suggested that common stem cells exist in adults for at least T, NK, and B cells. The use of *hprt* gene alterations as specific cellular markers provides a novel method for identifying stem cells in the lymphocyte lineage and for studying lymphocyte differentiation in humans.

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