1986 68: 378-385

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Use of Highly Polymorphic DNA Probes for Genotypic Analysis Following Bone Marrow Transplantation

By Robert G. Knowlton, Valerie A. Brown, Jeffrey C. Braman, David Barker, James W. Schumm, Christine Murray, Tak Takvorian, Jerome Ritz, and Helen Donis-Keller

The use of DNA markers known as restriction fragment length polymorphisms is a sensitive and informative method of distinguishing patient and allogeneic donor cells after bone marrow transplantation. To apply the test, it is necessary in each case to find DNA probes that display patient-specific and donor-specific bands in Southern transfer hybridization. We have isolated a set of 12 cloned DNAs from highly polymorphic loci by which siblings can usually be distinguished. With just four of these probes, we can expect to distinguish the genotypes of the recipient and a sibling donor in more than 99% of cases (except between identical twins). The availability of many highly polymorphic probes also allows selection of an optimal probe for each case, one that can detect both the patient and donor-specific bands in a single hybridization with maximum resolution and sensitivity. We have applied these probes to the analysis of cells from peripheral blood and bone marrow after transplantation and demonstrated their usefulness in confirming engraftment of donor cells or graft rejection, and in detecting mixed lympho-hematopoietic chimerism.

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BONE marrow transplantation is becoming the therapy of choice for a number of hematopoietic disorders and malignancies.[1-3] Because of the complex interactions among the host tissues, the donor immune system and the residual host immune system, analysis of the respective contributions of donor and recipient cells to the lympho-hematopoietic system is critical to interpretation of the events following transplantation.

The use of DNA sequence polymorphisms is an informative and versatile method of distinguishing patient and donor cells.[4-8] Phenotypically neutral sequence variations in the population can be identified as restriction fragment length polymorphisms (RFLPs), usually observed in Southern transfer hybridizations with cloned DNA probes.[9] Among different individuals, the restriction fragments homologous to a cloned DNA probe can occasionally differ in length, either because of mutations in restriction enzyme cleavage site or variation in the length of DNA between restriction sites as a result of insertions or deletions. Stably inherited as a part of an individual's genome and propagated to all cells, these DNA polymorphisms constitute a reliable set of markers for cells originating from a particular individual. Unlike other transplantation markers (eg, red blood cell antigens, immunoglobulin isotypes, karyotypes),[10-18] RFLP analysis is applicable to all hematopoietic cells except fully differentiated erythrocytes, in aggregate or in sorted cell fractions.[5,8]

The applicability of genotypic analysis to particular cases depends on discovery of informative DNA polymorphisms, ie, distinguishable alleles in the marrow donor and recipient. The likelihood that a particular probe will reveal distinct alleles in recipient and donor depends on the number of alleles and their distribution in the population. Most RFLPs are two allele variants of a single restriction site and therefore rarely informative in comparisons of patient and donor genotypes. One useful probe is the plasmid pDP34,[19] which hybridizes to distinct bands from the X and Y chromosomes and can always be used to detect the male genotypic pattern in sex mismatch cases. Analysis of cellular origin in most transplantation cases has depended primarily on the cloned DNA probe pAW101,[20] which identifies a locus with multiple RFLP alleles. However, because the donor and recipient of a transplant are siblings or very closely related, testing a single genetic marker, even a highly polymorphic DNA sequence, cannot guarantee that the two cell types can be distinguished. For no matter how many alleles exist in the population, each parent carries at most two different alleles, and two siblings have a 1/4 chance of inheriting the same two alleles of a polymorphic locus from their parents, and an additional 1/2 probability of sharing one parental homologue. This means that siblings will be indistinguishable by any single genetic marker in at least 25% of the cases. Probes for more than one highly polymorphic locus are therefore necessary to ensure detection of distinguishable patient and donor alleles. In this paper we describe a set of probes for highly polymorphic loci in the human genome that virtually guarantees the feasibility of determining the origin of nucleated cells in bone marrow transplants, even between closely related individuals.

The utility of these probes in analysis of transplant cases is demonstrated in this preliminary application to a set of leukemia patients who received bone marrow transplants from related allogeneic donors. Using genotypic analysis we have been able to distinguish patients with stable engraftment of donor cells exclusively, patients with chimeric cell populations associated with leukemic recurrence, and patients who appear to develop a stable chimeric condition.

MATERIALS AND METHODS

Patients. Cells were obtained prior to bone marrow transplantation from 10 patients and their related allogeneic donors. Each of those patients underwent marrow transplantation as treatment for hematologic malignancy. Eight patients received marrow from HLA-compatible siblings and two patients received marrow from partially incompatible related donors. In six instances patients and donors were of the same sex, but in four cases there was a sex mismatch. Transplantation and subsequent sampling of blood and

bone marrow were approved by the Institutional Review Boards of the Dana-Farber Cancer Institute and Brigham and Women's Hospital and were performed with the informed consent of the patients.

Cell samples. Peripheral blood and bone marrow samples from patients and donors were collected in preservative-free heparin and mononuclear cells isolated by Ficoll/Hypaque density sedimentation. In some instances, peripheral blood mononuclear cells were cultured in vitro following stimulation with either phytohemagglutinin (PHA) or allogeneic B-cell line (Laz 388). After this initial stimulation, cells were cultured in lymphocyte conditioned medium (LCM) containing IL-2. All cell samples were cryopreserved in 10% DMSO using standard techniques and stored in the vapor phase of liquid nitrogen.

Cellular DNA preparation. High molecular weight DNA was prepared from isolated nuclei obtained by centrifugation following solubilization of the cell suspension in Triton X-100. The nuclear pellet was resuspended in 1% SDS and treated with Proteinase K (Boehringer/Mannheim), 10 μg/mL, for 16 hours at 42 °C. After extraction with phenol and chloroform/isoamyl alcohol (24:1), DNA was precipitated with isopropanol (1.5 vol), redissolved in TE (10 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA), and precipitated with ethanol. DNA was redissolved in TE at a concentration of 250 μg/mL. Recovery of DNA from frozen cell samples was generally 5 micrograms per 10^6 cells.

Analysis of genomic restriction fragments. DNA samples at 175 μg/mL were digested to completion with restriction endonucleases (5 U/μg) from New England Biolabs, Beverly, Mass, in conditions recommended by the supplier. The extent of digestion was monitored in identical reaction mixtures containing both 1 μg of human DNA and 1 μg bacteriophage lambda DNA, stained with ethidium bromide after agarose gel electrophoresis; complete digestion was assumed if the lambda DNA band pattern represented a limit digest. Digested DNA samples were size fractionated by electrophoresis in 0.8% agarose gels, transferred to nylon membranes (Zeta-Bind, AMF Cuno, Meriden, Conn) in 25 mmol/L sodium phosphate, pH 6.5, according to Barker et al.[21] After prehybridizing the filters for 2 to 24 hours at 42 °C in 5 × SSC, 40 mmol/L phosphate, pH 7, 5 × Denhardt's solution, 100 μg/mL denatured salmon DNA, 10% dextran sulfate, and 50% formamide, ^{32}P-labeled probe was added (10^6 dpm/mL) to hybridize for 20 hours at 42 °C. After hybridization, filters were washed for 30 minutes at 20 °C with 2 × SSC, 0.1% SDS, and 60 minutes at 65 °C in 0.1 × SSC, 0.5% SDS. Autoradiography was carried out for one to five days at −70 °C with Kodak XAR-5 film and intensifying screens (DuPont Cronex Lightning-Plus).

Probes from polymorphic loci. These polymorphic DNA probes were isolated and characterized at Collaborative Research, Inc, for application to genetic linkage mapping.[22,23] Human genomic clones in bacteriophage Charon 4A were isolated from the library of Lawn et al.[24] Randomly chosen, single-copy phage clones were screened in Southern blot hybridizations to restriction digests from five individuals for restriction fragment length polymorphisms.[22] Twelve genomic clones with multiple allelic variations suitable for genotypic analysis of bone marrow transplants were selected for this study.

Recombinant bacteriophage DNA was prepared from plate lysates by the methods of Davis et al[25] and radioactively labeled by nick translation[26] with ^{32}P deoxynucleotide triphosphate (New England Nuclear, Boston) to a specific activity of 1 to 5 × 10^8 dpm/μg.

All work with recombinant bacteriophage was carried out under P1 conditions of containment in accordance with National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules.

RESULTS

Characterization of highly polymorphic DNA probes. We have obtained from a human genomic library a set of DNA clones that hybridize to sequences with extraordinary polymorphic variation.[23] Hybridization patterns of these probes to digests of DNA from a series of unrelated people show a spectrum of variable bands, with few individuals sharing the same pattern (Fig 1). The probes are randomly cloned single-copy sequences ranging in length from 11 to 17 kilobases inserted in the bacteriophage Charon 4A vector. Because the cloned DNA segments are large, the probes generally hybridize to several contiguous restriction fragments in the genomic digests. Each probe hybridizes to restriction fragments derived from a single chromosomal locus, as seen from the Mendelian inheritance pattern of the RFLPs in human pedigrees (data not shown).

For most of the probes described here, the same restriction fragment length variation among individuals can be detected with several different restriction enzymes, suggesting that the polymorphisms result from DNA sequence rearrangements at these loci rather than simple mutation of restriction sites. At some loci (detected with LAM4-159, LAM4-427, LAM-123, and LAM4-1214, for example), several different restriction fragments vary in length independently, with the result that each of the two alleles carried by an individual consists of multiple fragments potentially different from those of another individual. Fig 2 displays the allelic sets of PstI restriction fragments from the locus hybridizing to LAM4-159 as observed in chromosomes of 18 unrelated persons. Each locus consists of variable segments hybridized by the left side of the probe and by the right side of the probe, separated by fragments of constant size in the center. Each of the eight known variants of the left-side PstI fragment is associated with more than one of the eight observed right-side variants in different chromosomes, generating at least 16 allelic combinations already observed at this locus.

Not only does each of these loci exist in a large number of allelic forms, but the alleles are also dispersed in the population such that the probability that two parents will share the same alleles is low. This probability is typically expressed as the polymorphism information content, or PIC,[9] which is calculated from the allele frequencies and is a measure of the probability that the parental chromosome contributions can be determined in the offspring. Table 1 lists the estimated PIC values for each of our polymorphic probes; most are at least as informative as pAW101 (PIC = 0.85), and more informative than all but a few of the known polymorphic markers in the human genome.[9,27] From the number of alleles observed at each locus and their frequencies in the population, we can also calculate the likelihood that each probe will be informative in the special case of comparing genotypes of two siblings (Table 1). For the probes with the highest PIC values, the predicted probability of distinguishing siblings approaches the theoretical maximum of 75%.

Because the occurrence of distinguishable alleles at one locus is independent of the alleles at another locus, the application of the test can be extended to virtually all patient donor pairs by selecting from a set of highly polymorphic
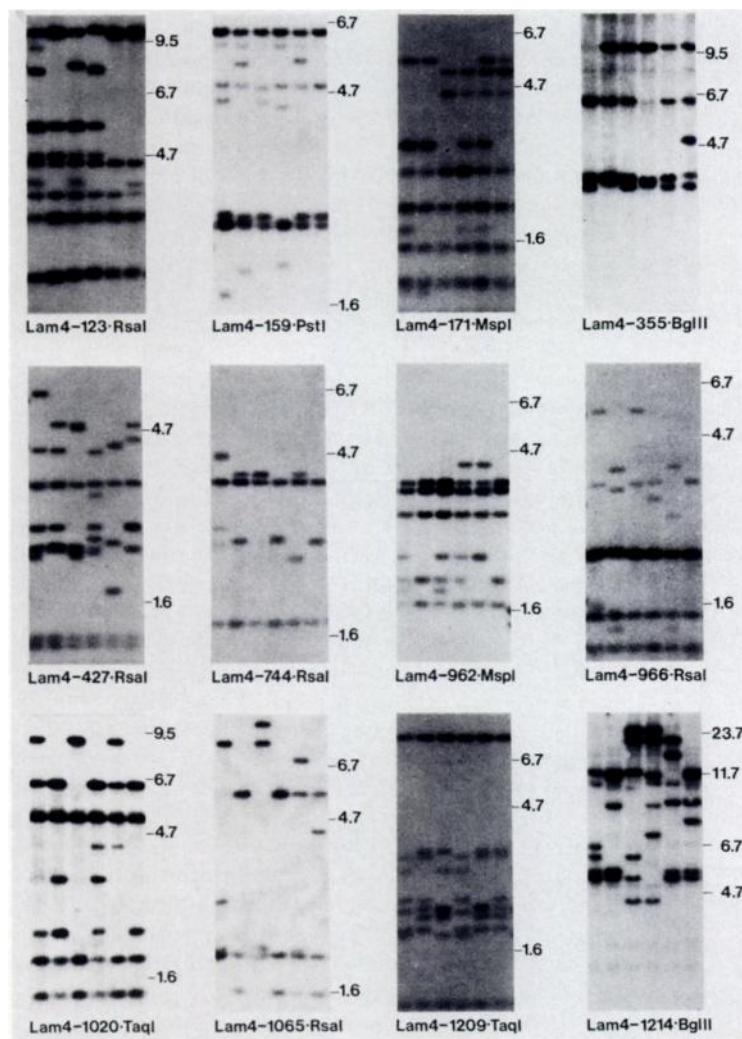
Fig 1. Restriction fragment patterns at highly polymorphic loci. DNA from 6 unrelated individuals was digested with restriction enzymes shown and hybridized to each cloned DNA probe as described in Materials and Methods. Migration of restriction fragment markers of known molecular weight is shown at right of each autoradiogram.

probes. In comparing sibling donor and recipient genotypes, this premise is valid if the polymorphic loci are not genetically linked, ie, if they are on different chromosomes or are separated with a recombination frequency approaching 50%. (A patient and donor who have inherited the same marker from a parent are also likely to have inherited the same DNA sequences in the surrounding region of that chromosome.) We have traced the inheritance of alleles of each of the polymorphic probes listed in Table 1 in several large, multigenerational families and compared their inheritance patterns for indication of genetic linkage. None of the probes is closely linked to any other with measured recombination frequency less than 25% (unpublished results). Application of each RFLP probe to a patient-donor pair therefore represents an independent test for genetic heterogeneity. The probability of genetic identity at all loci tested is therefore the product of the probability of identical alleles with the individual probes (Table 1). With all of the 12 probes used in this study, the probability that two siblings would be identical is only 1.3×10^{-6}. In fact, we can be quite confident of distinguishing siblings with only four of the most polymorphic probes (LAM4-427, 1214, 355, and 159). Genotypic comparisons at just these loci would be informative in more than 99% of the cases.

Identification of informative probes in transplant cases. Genotypic analysis of cellular DNA in bone marrow transplantation proceeds in two stages. First, DNA samples of donor and recipient origin are tested with probes from highly polymorphic loci to determine for each locus whether the two genotypes are distinguishable in Southern transfer-hybridization. It is necessary to find both donor and patient-specific bands in the hybridization patterns, so that contributions of both genomes can be distinguished in a mixture of the two. The ideal probe would hybridize to both donor-specific and patient-specific bands in the same restriction digest, so that both genotypes could be detected in the same sample and hybridization.

Probes that fulfill these criteria for informative and efficient genotypic analysis were selected from the panel to apply to ten bone marrow transplant cases. First, the genotypes of the ten transplant recipients and their respective donors were compared by Southern blot hybridization patterns of the 12 probes listed in Table 1. Restriction fragment patterns, such as those shown in the autoradiograms in Fig 3,
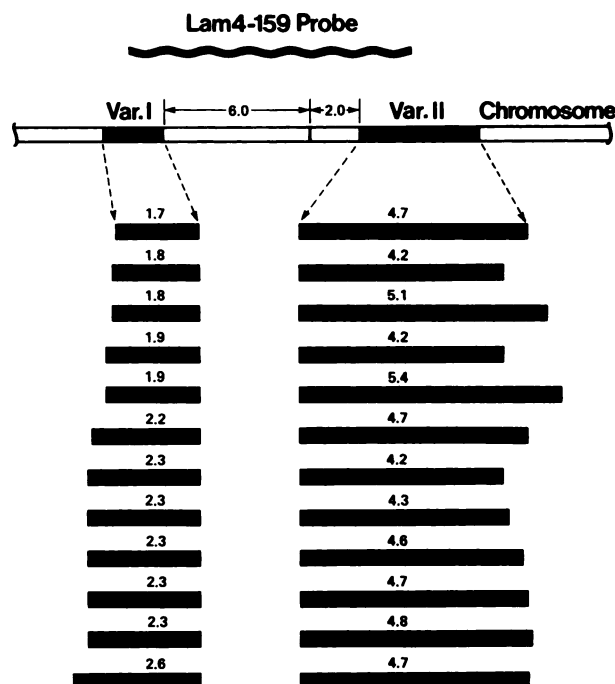
Lam4-159 Probe

Fig 2. Variable fragment lengths at locus defined by LAM4-159. Four contiguous PstI fragments are detected with the phage clone LAM4-159, two fragments of constant length (6 kb and 2 kb) flanked by two fragments of varying length in different alleles. The length variation in fragment I (Var.I) is apparently independent of the variation in fragment II (Var.II), so that the number of different combinations is large. The examples of LAM4-159 alleles shown are 12 different fragment combinations observed among 36 chromosomes of 18 unrelated individuals.

were scored for the presence of patient-specific and donor-specific bands. As expected, each patient and donor genotype could be distinguished by restriction fragments at several of the probed loci (Table 2). Because of the highly polymorphic character of the genetic loci tested, each probe was informative for several patient-donor pairs. In general, the probes with the highest PIC values were the most successful at distinguishing between patient and donor genotypes. Having identified several informative probes in each transplant case, we chose one probe that was expected to provide the most sensitive and reliable measurement of proportions of each genotype in posttransplant samples. Sensitivity is maximal when bands to be detected show strong hybridization signals, are well separated from other bands, and background hybridization is low. Furthermore, some probes hybridize to more than one patient-specific or donor-specific band in a single digest, providing opportunities for repeated measurement of the proportions of the two DNAs in the same sample (eg, Fig 3A, patient-donor pair #6).

Analysis of cell populations after transplantation. Cell samples taken after bone marrow transplantation were analyzed with the polymorphic probes chosen for each patient-donor pair. Hybridization patterns of posttransplant DNA samples were compared with the patterns of pure donor and recipient DNA in adjacent lanes (Figs 4 and 5). The fraction of the total cell population represented by either donor cells

or patient cells was estimated from the respective band intensities in the autoradiogram. Adjacent control lanes contained admixtures of known proportions of patient and donor DNA and served as standards for making quantitative estimates of these DNAs in posttransplant samples. Direct comparison of patient and donor bands in the same hybridization is critical to quantitative measurement of the proportions of the two genotypes, because the intensity of bands can vary substantially depending on the efficiency of transfer of the DNA to the filter, the specific activity of the radioactive probe, and the stringency of hybridization and washing. In particular, it is important to establish the sensitivity of the assay when the autoradiogram shows only the donor genotypic pattern in order to determine the maximum level of cells of patient origin that could have escaped detection. In these hybridizations with the recombinant bacteriophage DNAs as probes, we could detect cellular DNA that was present as 5% to 10% of the total. Elimination of the lambda vector sequences from the same probes can improve the sensitivity to approximately 1% (results not shown).

Results demonstrating successful engraftment of donor cells in patient #1 are shown in Fig 4A. DNA was prepared from peripheral blood taken at 16 to 20 months after transplant, as well as from bone marrow aspirate collected 17 months posttransplant. After restriction enzyme digestion and transfer hybridization with probe LAM4-1214, the restriction fragment patterns of these DNA samples were compared with the pretransplant patient and donor patterns. The presence of the 25 kb, 9.3 kb, and 6 kb bands of donor genotype and the absence of 7.5 kb and 5.6 kb bands characteristic of patient genotype confirms the successful

Table 1. Polymorphic DNA Probes and Their Expected Information Value*

Probe	Enzyme	PIC	Probability Siblings Distinguishable
LAM4-427	Rsal	0.95	0.72
LAM4-1214	BglII	0.94	0.72
LAM4-355	BglII	0.90	0.70
LAM4-159	PstI	0.90	0.70
LAM4-966	Rsal	0.88	0.69
LAM4-123	Rsal	0.81	0.65
LAM4-744	Rsal	0.80	0.65
LAM4-1020	Taql	0.80	0.65
LAM4-1065	Rsal	0.75	0.62
LAM4-1209	Taql	0.75	0.62
LAM4-171	MspI	0.70	0.60
LAM4-962	MspI	0.70	0.60

*PIC is the polymorphism information content, the probability of informative segregation of alleles in a given family at the marker locus.[16] The probability that two siblings will be identical at the locus defined by each probe is also calculated from the allele frequencies in the population:

Probability of sibling identity $= \dfrac{1}{4} + \dfrac{1}{2}\sum_{i=1}^{n} f_i^2 + \dfrac{1}{2}\sum_{i=1}^{n}\sum_{j=1}^{n} f_i^2 f_j^2 \approx \dfrac{1}{4} + \dfrac{1}{2}\sum_{i=1}^{n} f_i^2$

where f_i and f_j are the frequencies in the population of alleles i and j, and n is the total number of alleles. The probability that the probe will distinguish two siblings is then one minus the probability that they have the same two alleles.
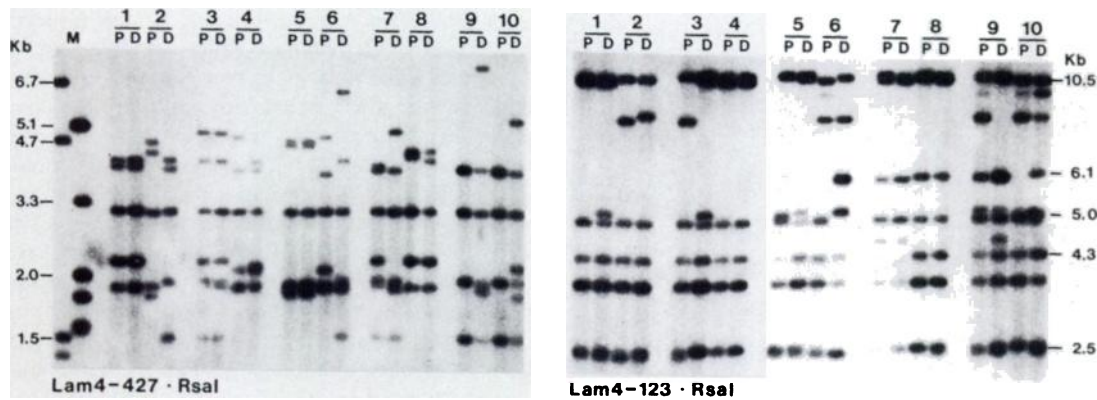
Fig 3. Identification of patient and donor-specific bands in DNA hybridization before transplantation. Pretransplant genomic DNA samples (2 µg) from 10 patient/donor pairs were digested with RsaI and hybridized with RFLP probes LAM4-427 and LAM4-123. Patient and donor-specific fragments can be detected in all 10 cases with more than one of the 12 probes tested.

engraftment. The minimum proportion of recipient cells that could have been detected is approximately 5% in this assay, as seen in the control samples in adjacent lanes. The conclusion that stem cell ablation was complete and donor-cell engraftment was successful is particularly strengthened by this genotypic assay since all the cell populations sampled, both peripheral blood and bone marrow, are free of detectable patient cells.

The opposite result was found in the case of patient #3, the recipient of a partial HLA mismatched marrow transplant. Phenotypic and functional analysis of peripheral blood mononuclear cells in this patient suggested that immunologic rejection of donor cells had occurred. This conclusion was supported by the RFLP analysis with LAM4-1214 (Fig 4B) which showed that all (>90%) of the nucleated cells recovered from peripheral blood drawn at 14 days after transplant and cultured in vitro were of host genotype.

One example of lympho-hematopoietic chimerism is shown in Fig 5A. Patient #2 is a stable transplant recipient whose chimeric condition was detected by red cell antigen typing and karyotype analysis. Retrospective analysis of peripheral blood DNA with RFLP probe LAM4-427 demon-

strates the progressive repopulation with cells of recipient origin. Peripheral blood cells were exclusively of donor type until 4 months after transplantation, when cells of recipient genotype were first detected at the 10% level. In subsequent months, the proportion of host cells in peripheral blood and in bone marrow has increased to approximately 50%. Despite partial reconstitution with recipient cells, there has been no evidence of recurrence of leukemic cells.

Another example of patient/donor chimerism is shown in Fig 5B (patient #9). By RFLP analysis with probe LAM4-123, exclusive engraftment with donor cells was seen 2 months after transplant. At 5 months posttransplant, peripheral blood cells were 20% of patient origin by RFLP analysis. Leukemic relapse was clinically evident 6 months after transplant, and by this time, the proportion of patient cells as measured by RFLP analysis had risen to 75% in both peripheral blood and bone marrow.

DISCUSSION

Determination of the origin of cells in the lympho-hematopoietic system will be useful for interpreting the course of

Table 2. DNA from 10 Patient-Donor Pairs Was Tested by Hybridization With Each of the 12 Probes Listed in Table 1 and Scored for the Presence of Patient-Specific (P) and Donor-Specific (D) Bands

Probe	Patient/Donor Pair										Number of Cases Informative
	1	2	3	4	5	6	7	8	9	10	
LAM4-427	—	**PD**	—	PD	—	PD	PD	PD	D	D	7
LAM4-1214	**PD**	P	**PD**	PD	—	**PD**	**PD**	D	—	**PD**	8
LAM4-355	PD	D	P	D	—	PD	PD	—	—	—	6
LAM4-159	PD	P	PD	—	PD	—	—	—	—	—	4
LAM4-966	—	PD	D	P	PD	P	PD	—	PD	—	7
LAM4-123	D	PD	PD	—	PD	PD	P	—	**PD**	D	7
LAM4-744	—	—	P	P	PD	PD	P	—	PD	P	7
LAM4-1020	—	PD	D	—	PD	—	D	—	D	P	6
LAM4-1209	—	—	PD	—	PD	—	—	—	PD	—	3
LAM4-1065	—	—	PD	**PD**	**PD**	D	P	**PD**	—	P	7
LAM4-171	—	—	P	—	P	—	—	P	—	D	4
LAM4-962	D	D	—	P	P	—	—	PD	PD	—	6
Total	5	7	9	6	9	7	7	5	5	7	

PD, both patient and donor-informative bands observed in the same DNA hybridization; —, no informative bands. Symbols in boldface indicate the DNA probe chosen for posttransplant analysis of each case.
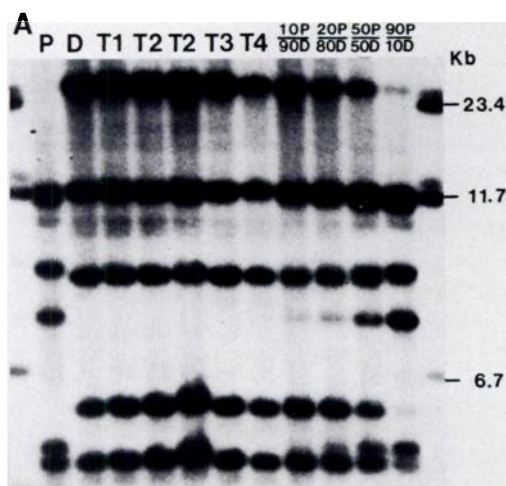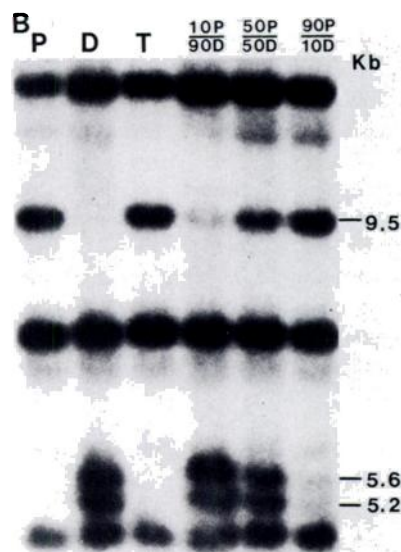
Fig 4. Monitoring engraftment with patient and donor-specific restriction fragments. (A) Successful engraftment in patient #1 is demonstrated in DNA hybridization analysis with probe Lam4-1214 by the persistence of donor-specific fragments (25 kb, 9.3 kb, and 6.0 kb) and the absence of the patient-specific fragment (7.5 kb and 5.6 kb) up to 20 months following transplantation. Faint bands at 10.5 kb and 15 to 20 kb reflect cross-hybridization of the probe with other loci and are not used in diagnosis. P and D indicate pretransplant patient and donor DNA's. T1 to T4 are posttransplant patient DNA samples: T1, peripheral blood (PB) 16 months post-BMT; T2 (2 lanes), PHA-stimulated cultured PB and anti-T12-treated[29] cultured PB, both 17 months post-BMT; T3, bone marrow aspirate, 17 months post-BMT; T4, PB 20 months post-BMT. (B) Failure of engraftment in patient #3. Lane T contains DNA from cultured peripheral blood cells drawn 14 days after transplant. Admixtures of DNA from donor and pretransplant patient blood samples are included as internal standards; eg, 10P/90D indicates 10% patient DNA and 90% donor DNA in the sample. All lanes contain 4 μg of DNA.

leukemic cells after transplantation,[6] and in rare cases, to identify tumors of donor origin.[4,7] Ginsburg et al[5] have detected lympho-hematopoietic chimerism in transplant patients treated for aplastic anemia, SCIDS, and leukemia, and demonstrated different proportions of patient and donor cells in different cell types of a single individual. The results so far have shown the power of RFLP analysis to assay cell populations for the fractions of patient and donor cells with high sensitivity, and also to test cell populations inaccessible with more conventional assays. The results we present here provide further demonstration of the utility of genotypic
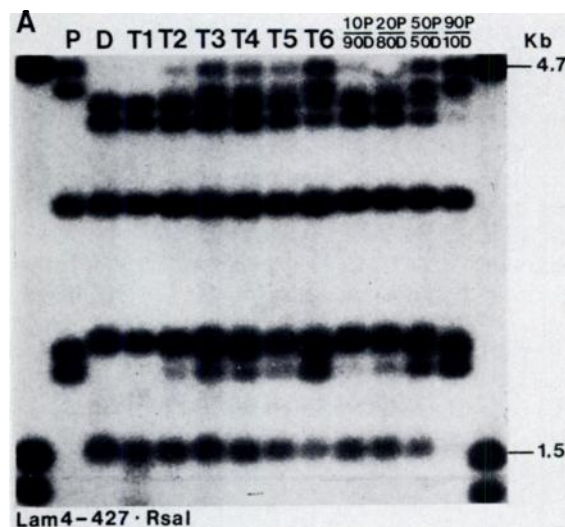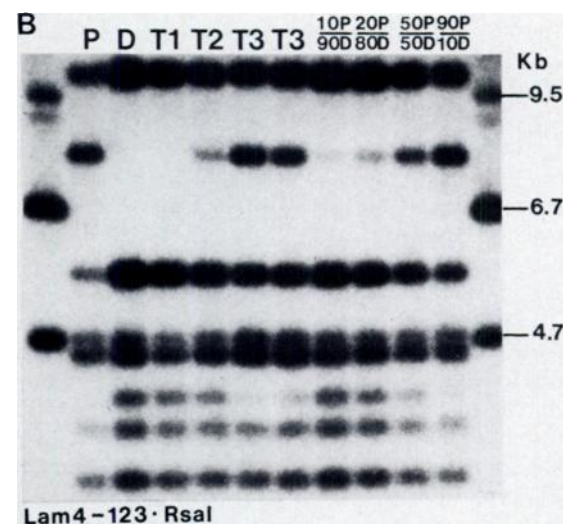
Fig 5. Lympho-hematopoietic chimerism. (A) Engraftment of donor marrow in patient #2 is followed by reappearance of cells of patient origin. P and D, pretransplant patient and donor DNAs, respectively; T1, cultured PB, 3 months post-BMT; T2 to T5, PB at 4, 7, 9, and 12 months post-BMT; T6, bone marrow at 12 months post-BMT. (B) Reappearance of cells of recipient origin in patient #9 after engraftment of donor cells. T1, cultured PB drawn 2 months post-BMT; T2, PB at 5 months post-BMT; T3, PB (left) and bone marrow (right) at 6 months post-BMT. All lanes contain 4 μg of DNA. Proportions of cells of patient and donor genotype in samples showing mixed chimerism are estimated by comparison with mixtures of known composition in adjacent lanes.

events following bone marrow transplantation. The use of DNA polymorphisms for this purpose has been demonstrated to be an informative and sensitive method in several applications. For instance, patient-specific RFLP patterns have been used to confirm the host origin of recurrent

analysis to detect mixed chimerism in peripheral blood. In addition, we have applied the test to cells in bone marrow aspirates and found that the proportion of patient and donor cells in peripheral blood reflects the composition of bone marrow cells in several cases. This capability of analyzing any nucleated cell population makes the RFLP test a versatile research tool.

We have increased the usefulness of DNA genotype testing by collecting a set of highly polymorphic probes and making the routine testing of virtually all bone marrow transplant patients feasible. The limitation to RFLP analysis with probes currently used has been the uncertainty of finding adequate patient and donor-specific alleles with any given probe. Because screening probes against pretransplant samples of recipient and donor DNA to find distinguishing bands is the most laborious part of the procedure, probes likely to reveal different alleles are required to make the search practical. Furthermore, because siblings frequently coinherit the same alleles, more than one highly polymorphic probe must be available to screen for informative alleles. Even the highly polymorphic probe, pAW101,[20] with more than eight possible alleles is expected to be uninformative in one third of cases with sibling donors (see Table 1). However, if several highly polymorphic probes are tested, informative markers can be found for virtually all patient-donor pairs except identical twins. We have described here the application of 12 highly polymorphic probes with PIC values ranging from 0.7 to 0.95. Each of the 12 probes was found to be informative in at least three and as many as eight of the ten transplant cases examined, a success rate consistent with the number and frequency of alleles at each of the loci. The net result is that detection of both recipient and donor cells was possible in each case with any of several probes available. We are therefore confident of our ability to find informative alleles for all patients and allogeneic donors. Routine analysis employing only a subset of these probes would be practical and informative in almost all cases.

The advantages of highly polymorphic probes for bone marrow transplant analysis go beyond the probability of discovering informative alleles. Another consequence of the extraordinary variability of these genetic markers is that both patient and donor-specific bands are often displayed with a single probe in a single DNA restriction digest. In this situation, not only is the procedure simplified, but quantitative estimates of proportions of the two cell types are far more reliable. The patient and donor-specific band intensities can be compared directly in the same hybridized sample, without introducing errors due to experimental variation in DNA concentration, gel transfer, or probe hybridization. Of those probes informative for both genotypes, it is generally possible to choose one that displays hybridization patterns suitable for resolution and detection of trace amounts of patient or donor DNA in a mixture of the two. In the optimal pattern, the patient and donor-specific bands are strongly hybridizing and well-resolved by agarose gel electrophoresis. One drawback to the commonly used probe pAW101 is that the allelic EcoRI fragments that must be distinguished are as long as 25 kilobases, at the limit of resolution of agarose gels.

We can also choose several probes to facilitate the routine application of the test by reducing the number of different restriction enzyme digests and gel transfers. As can be seen in Table 1, the polymorphic alleles of the majority of these probes are displayed with only a few different restriction enzymes. By digesting pretransplant samples of recipient and donor DNA with only two enzymes, RsaI and BglII, we can test up to seven of our most highly polymorphic probes and be confident of identifying a probe useful for diagnosis. If for any reason the patient DNA sample is limited in quantity (eg, from an infant or leukopenic patient), the same samples digested with RsaI or BglII could be tested against all seven of these probes by multiple uses of the hybridization filter. Performance of the test requires less than 10μg of DNA per sample, which can generally be obtained from 1 mL of blood.

Examination of the data we have obtained by RFLP analysis of posttransplant samples supports the contention that the test is more sensitive and more reliable than other available methods. In each case in which mixed chimerism developed, the reappearance of patient cells was detected through patient-specific alleles in DNA samples taken at least as early as the first signs of chimerism by karyotype analysis or red blood cell antigens, and in some instances, in advance of other indications. Furthermore, the relative hybridization signals of patient and donor bands give a quantitative measurement of the proportions of the two genotypes. Progressive shifts in the proportion of patient and donor cells were clearly demonstrated in the DNA hybridizations.

We expect that the routine performance of genetic typing of lymphoid and hematopoietic cells will contribute substantially to our understanding of cellular interactions following bone marrow transplantation. In particular, the determination of host or donor origin of cell populations should be pertinent to evaluating graft rejection, graft-versus-host disease, and recurrence of leukemia. The test can also provide the clinician an accurate measure of the completeness of donor engraftment. We have shown that by using a set of highly polymorphic probes we can efficiently perform the diagnosis in virtually all allogeneic transplant cases, so that routine application of the test is feasible.

ACKNOWLEDGMENT

REFERENCES

1. Good RA, Kapoor N, Reisner Y: Bone marrow transplantation—An expanding approach to treatment of many diseases. Cell Immunol 82:36, 1983

2. Thomas ED, Clift RA, Storb R: Indications for marrow transplantation. Ann Rev Med 35:1, 1984

3. Santos GW: Bone marrow transplantation in leukemia. Cancer 54:2732, 1984

4. Schubach WH, Hackman R, Neiman PE, Miller G, Thomas ED: A monoclonal immunoblastic sarcoma in donor cells bearing Epstein-Barr virus genomes following allogeneic marrow grafting for acute lymphoblastic leukemia. Blood 60:180, 1982

5. Ginsburg D, Antin JH, Smith BR, Orkin SH, Rappeport JM: Origin of cell populations after bone marrow transplantation. J Clin Invest 75:596, 1985

6. Minden MD, Messner HA, Belch A: Origin of leukemic relapse after bone marrow transplantation detected by restriction fragment length polymorphism. J Clin Invest 75:91, 1985

7. Witherspoon RP, Schubach W, Neiman P, Martin P, Thomas ED: Donor cell leukemia developing six years after marrow grafting for acute leukemia. Blood 65:1172, 1985

8. Blazar BR, Orr HT, Arthur DC, Kersey JH, Filipovich AH: Restriction fragment length polymorphisms as markers of engraftment in allogeneic marrow transplantation. Blood 66:1436, 1985

9. Botsein D, White RL, Skolnick M, Davis RW: Construction of a genetic linkage map using restriction fragment length polymorphisms. Am J Hum Genet 32:314, 1980

10. Thomas ED, Buckner CD, Banaji M, Cliff RA, Fefer A, Flournoy N, Goodell BW, Hickman RO, Lerner KG, Neiman PE, Sale GE, Sanders JE, Singer J, Stevens M, Storb R, Weiden PL: One hundred patients with acute leukemia treated by chemotherapy, total body irradiation and allogeneic marrow transplantation. Blood 49:511, 1977

11. Blume KG, Beutler E, Bross KJ, Schmidt GM, Spruce WE, Teplitz RL: Genetic markers in human bone marrow transplantation. Am J Hum Genet 32:414, 1980

12. Branch DR, Gallagher MT, Forman SJ, Winkler KJ, Petz LD, Blume KG: Endogenous stem cell repopulation resulting in mixed hematopoietic chimerism following total body irradiation and marrow transplantation for acute leukemia. Transplantation 34:226, 1982

13. Bross KJ, Schmidt GM, Blume KG, Spruce WE, Farbstein MJ: Confirmation of bone marrow engraftment by demonstration of blood group antigens on red cell ghosts. Transplantation 28:257, 1979

14. Witherspoon RP, Schanfield MS, Storb R, Thomas ED, Giblett ER: Immunoglobulin production of donor origin after marrow transplantation for acute leukemia or aplastic anemia. Transplantation 26:407, 1978

15. Sparkes RS, Sparkes MC, Gale RP: Immunoglobulin synthesis following allogeneic bone marrow transplantation in man. Transplantation 27:212, 1979

16. Borgaonkar DS, Bias WB, Sroka BM, Hutchinson JR, Santos GW: Identification of graft and host cells in bone marrow transplants by the quinacrine technique of chromosome identification. Acta Cytol 18:263, 1974

17. Sparkes MC, Crist ML, Sparkes RS, Gale RP, Feig SA, UCLA Transplantation Group: Gene markers in human bone marrow transplantation. Vox Sang 33:202, 1977

18. Lawler SD, Baker MC, Harris H, Morgenstern GR: Cytogenetic studies on recipients of allogeneic bone marrow using sex chromosomes as markers of cellular origin. Br J Haematol 56:431, 1984

19. Page D, De Martinville B, Barker D, Wyman A, White R, Francke U, Botstein D: Single-copy sequence hybridizes to polymorphic and homologous loci on human X and Y chromosomes. Proc Natl Acad Sci USA 79:5352, 1982

20. Wyman AR, White R: A highly polymorphic locus in human DNA. Proc Natl Acad Sci USA 77:6754, 1980

21. Barker D, Holm T, White R: A locus on chromosome 11p with multiple restriction site polymorphisms. Am J Hum Genet 36:1159, 1984

22. Schumm JW, Knowlton RG, Braman JC, Barker D, Vovis G, Akots G, Brown V, Gravius T, Helms C, Hsiao K, Rediker K, Thurston J, Botstein D, Donis-Keller H: Detection of more than 500 single copy RFLP's by random screening. 8th Human Gene Mapping Workshop, Cytogenet Cell Genet 40:739, 1985

23. Braman JC, Barker D, Schumm JW, Knowlton RG, Donis-Keller H: Characterization of very highly polymorphic RFLP probes. 8th Human Gene Mapping Workshop. Cytogenet Cell Genet 40:489, 1985

24. Lawn RM, Fritsch EF, Parker RC, Blake G, Maniatis T: The isolation and characterization of a linked δ- and β-globin gene from a cloned library of human DNA. Cell 15:1157, 1978

25. Davis RW, Botstein D, Roth JR: Advanced Bacterial Genetics. New York, Cold Spring Harbor Laboratory, 1980

26. Rigby PWJ, Dieckmann M, Rhodes C, Berg P: Labeling deoxyribonucleic acid to high specific activity by nick translation with DNA polymerase I. J Mol Biol 113:237, 1977

27. Willard H, Skolnick M, Pearson P, Mandel JL: Human gene mapping by recombinant DNA techniques. Eighth Human Gene Mapping Workshop. Cytogenet Cell Genet 40:360, 1985

28. Rohatiner A, Gelber R, Schlossman SF, Ritz J: Depletion of T cells from human bone marrow using monoclonal antibodies and rabbit complement: A quantitative and functional analysis. Transplantation (in press)