Monoclonality in B-Lymphoproliferative Disorders Detected at the DNA Level

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A new method was developed for detection of monoclonality at the DNA level in B-lymphoproliferative disease using the polymerase chain reaction and consensus primers for the V and J regions of the immunoglobulin gene. Monoclonality was detected in DNA from 15 of 15 clonal B-lymphoblastoid cell lines and from 19 of 23 cases of B-lymphocyte neoplasia, but not from any of 16 normal

**Laboratory Diagnosis** of lymphoproliferative diseases is conventionally based on histology or cytology, often supplemented by immunophenotyping. Recently, gene probing to detect rearrangement of the immunoglobulin (Ig) or T-cell receptor genes has been shown to be of value in some cases.1-5 as it can demonstrate monoclonality, assign a disorder to either the B- or T-lymphocyte lineage, and enable the detection of small numbers of neoplastic cells.

However, gene probing does have some disadvantages. The technique is complex, requires a large amount of DNA, and the time of 1 to 2 weeks to obtain the final result greatly decreases its practical value. This report describes a new method for detecting rearrangement of the Ig heavy chain gene based on DNA amplification using the polymerase chain reaction (PCR).6 The simplicity, speed, and flexibility of the method are likely to make it valuable in a variety of clinical situations.

**Materials and Methods**

**Principles of the Technique.** The technique is based on four principles: (1) The PCR will exponentially amplify a defined segment of DNA. Amplification by PCR requires the use of two oligonucleotide primers that bind specifically to homologous regions at the ends of the DNA segment of interest. The primer binding sites need to be relatively close to one another, of the order of 2 kilobases or less. (2) During lymphocyte differentiation, the variable (V), diversity (D), joining (J), and constant (C) regions of the molecule are recombined, so that there is a relatively short distance, approximately 400 base pairs (bp), between the beginning of the V region and the end of the J region.7 (3) Although the nucleotide sequences of the V, D, and J regions vary, there are sufficient conserved sequences within these regions to enable the construction of consensus oligonucleotide primers.8 The relatively conserved sequences include the framework regions of the V regions and portions of the D and J regions. (4) Nucleotides are randomly removed and inserted at the V-D and D-J junctions.9 As a consequence, the distance between two points situated outside the recombination junctions, such as the primer binding sites, varies from molecule to molecule. Thus, the lengths of DNA fragments amplified using the PCR and consensus primer will vary between the different Ig molecules produced by different B lymphocytes.

If a PCR is performed using consensus oligonucleotide primers for the V and J regions, then the results of amplification will depend on the cell population being studied. A population of nonlymphoid cells or T lymphocytes, in which the Ig gene is in the germ line configuration, will not show amplification of an Ig DNA fragment, as the V and J regions are too far apart. A population of B lymphocytes will show amplification of DNA as the Ig gene has been rearranged. Monoclonality of B lymphocytes will be characterized by amplification showing fragment length homogeneity, ie, a discrete band of amplified DNA on electrophoresis, whereas polyclonality will be characterized by DNA fragment length heterogeneity, ie, a broad band or smear on electrophoresis. The criterion for monoclonality at the DNA level is thus analogous to the criterion for monoclonality of Ig at the protein level.

**Technique.** PCR was performed as described by Saiki et al.10 using *Thermus aquaticus* (Tag) polymerase, 1 μg of DNA, and 30 to 40 cycles of amplification involving 2 minutes each of sequential denaturation at 95°C, annealing at 55°C, and extension at 72°C. Control tubes containing no DNA or a known DNA sample containing a rearranged Ig gene were set up with each experiment. The primers were synthesized on a Applied Biosystems 381A DNA Synthesizer (Applied Biosystems, Foster City, CA) and their sequences were: for the third framework portion of the V region: 5' ACACGGC(C/T)G(G/C)TGTTATCTGT 3' (termed Fr3A); for the J region: 5' TGAGG AGACG GTGACC 3' (termed LJI); or 5' GTGACC AGGG TN CCTGGCC CCG 3' (termed VLJH). FR3A is based on a consensus sequence for codons 89 through 95 from 17 human V regions, while LJH and VLJH are based on consensus sequences for codons 109 through 113 and 103 through 110 from the six J regions.11 These primers would be expected to generate a fragment of approximately 100 to 120 bases in length. The details of the design of the primers are discussed elsewhere.12

The amplified material was electrophoresed in 2% agarose and was visualized under ultraviolet light after ethidium bromide staining.

**Normal lymphocytes.** Polyclonal populations of peripheral blood lymphocytes (PBL) were obtained by Ficoll-Hypaque separation of peripheral blood. Monoclonal populations of nonneoplastic B lymphocytes were obtained by transforming mass populations of PBL with Epstein-Barr virus and then cloning at limiting dilution. Monoclonal populations of normal T lymphocytes were obtained by direct cloning of PBL at limiting dilution. DNA was extracted by treating cells with sodium dodecyl sulfate-proteinase K followed by phenol-chloroform extraction13 and, for each cloned population of B or T lymphocytes, monoclonality was confirmed by gene probing of the Ig or T-cell receptor genes. Fifteen B lymphocyte clones, 16 T lymphocyte clones, and 20 polyclonal PBL samples were studied.

**Patients with lymphoproliferative disorders.** A study was made of 14 patients with B-lymphocyte non-Hodgkin's lymphoma (B-
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NHL), 9 patients with B-lymphocyte chronic lymphocytic leukemia (B-CLL), 8 patients with T-lymphocyte NHL (T-NHL), 1 patient with T-lymphocyte CLL (T-CLL), and 8 patients with nonneoplastic reactive lymph node enlargement. DNA was extracted from freshly frozen involved tissue, usually lymph node, or sometimes marrow or blood. All of the patients had previously been studied by routine histology, immunohistochemistry, and Southern blotting of the Ig and T-cell receptor genes, and the disorder had been assigned to the B- or T-lymphocyte class.

RESULTS

Similar results were obtained using WH and VWH as primers for the J region; the latter gave slightly better amplification in some experiments and, as expected, resulted in amplified material slightly shorter in length.

A discrete amplified band 100 to 120 bp in length was seen in all 15 normal B-lymphocyte clones studied and in none of the 16 normal T-lymphocyte clones. Two amplified bands were seen in 3 of the 15 B-lymphocyte clones and presumably corresponded to two rearranged Ig genes. Each of the 20 polyclonal lymphocyte populations generated either no visible product or a heterogeneous collection of amplified fragments that were evident as a diffuse band of stained material in the gel.

One or two discrete bands of amplified DNA, 100 to 120 bp in length, were seen after PCR in 12 of 14 cases of B-NHL, 7 of 9 cases of CLL, 0 of 8 cases of T-NHL, 0 of 1 case of T-CLL, and 0 of 8 cases of reactive lymphadenopathy. Examples of the findings are shown in Fig 1. Discrete amplification in two cases of B-NHL and two cases of B-CLL could not be obtained despite changing a number of variables in the PCR. These variables included the annealing time or the concentration of Mg, primers, or Taq polymerase. The cases of reactive lymphadenopathy showed either no visible product or a diffuse band of amplified material indicating DNA fragment length heterogeneity. The four node aspirates from cases of B-NHL all showed a discrete band.

In 10 cases a discrete band amplified by the primers Fr3A and LJH was probed by VLJH after Southern transfer. The sequence VLJH lies 5' to that for LJH, and in each case hybridization to this Ig-specific sequence was demonstrated. The sensitivity of the technique in detecting a monoclonal population admixed with a polyclonal population was studied in three experiments by mixing cells from a neoplastic B-lymphocyte line, NALM-6, with different proportions of normal PBLs. DNA was extracted by boiling.11 The limit of detection of a discrete amplified band from the neoplastic population was 10%, 5%, or 1% in the three experiments, respectively.

DISCUSSION

Monoclonality of B lymphocytes, as evidenced by DNA fragment length homogeneity, was detected in 83% of cases of B-lymphoproliferative disease, but never in T-lymphoproliferative disease or in reactive disorders. The demonstration of monoclonality strongly suggests the diagnosis of neoplasia and the present technique is likely to be valuable in the diagnosis of B-lymphoproliferative disease and the distinction from reactive disorders. The exact value and the limitations of the technique will require the study of much larger numbers of patients, but the parallels between detection and interpretation of monoclonality at the DNA level and detection and interpretation of monoclonality at the protein level using serum electrophoresis are likely to be close.

Although there is no formal proof, the evidence is quite strong that the material amplified by the primers is specifically part of the Ig molecule. The primers were designed using sequences from the Ig gene and the amplified material

Fig 1. Gel electrophoresis after PCR amplification of DNA from 4 cases of B-lymphoproliferative disease (tracks 2, 4, 9, and 10), 2 cases of T-lymphoproliferative disease (tracks 3 and 8), and 2 reactive lymph nodes (tracks 5 and 11). Track 1 is marker DNA; track 7 is DNA from a B-leukemia cell line, which acted as a positive control; and track 6 is a negative control in which DNA had been omitted from the amplification reaction.
is of the appropriate size; amplified material from two leukemia lines has been sequenced and, at the 5' end, shows homologies with the 3' end of framework 3, and at the 3' end shows a J4 sequence in one case and a J6 sequence in the other case. The amplified material hybridizes to an integral Igspecific sequence, and the differences in size of material amplified by LJH and VLJH can be readily explained by binding of these two primers to different portions of the J region. Of equal importance for assessing specificity is the biologic observation that discrete amplified bands were seen only with known monoclonal B-lymphocyte populations and not monoclonal T-lymphocyte populations, and that broad bands were observed with known polyclonal lymphocyte populations.

The explanation for the failure to detect monoclonality in all cases of B-lymphoproliferative disease is not clear. Another index gene, the ras gene, was amplified by PCR in the specimens that did not show amplification of the Ig gene, indicating that the PCR was not being inhibited. Amplification of a discrete rearranged DNA fragment could not be produced by use of primers for other sequences of the Ig molecule; by variation of the reactants and conditions by the PCR so as to alter sensitivity or specificity of the reaction; or by restriction enzyme digestion, boiling, and gel filtration before the reaction. The specimens did not consist of a small number of neoplastic lymphocytes admixed with a large number of normal lymphocytes, as the histologic or cytologic specimens were reviewed to exclude this possibility, and in all cases rearrangement of the Ig gene had been detected by gene probing. One possible explanation for the false negative results is an unusual rearrangement, such as an inversion, of the Ig genes.

The study of rearrangements of the Ig or T-lymphocyte receptor gene using Southern blotting is of diagnostic value in a proportion of cases of leukemia or lymphoma, but the expense and time required to obtain a result greatly decrease the value of the approach. By contrast the present technique is simple and rapid, with results being obtainable within 24 to 36 hours. A further potential advantage is the extent of DNA amplification afforded by the PCR, which enables a much smaller amount of starting DNA to be used. This makes possible the study of needle aspirates or cytologic specimens, thus greatly increasing the flexibility and scope of the approach. The present PCR technique was shown to detect approximately 2% to 5% of a monoclonal population admixed in a larger polyclonal population. Below this level it was not possible to detect the discrete band of amplified monoclonal DNA separately from the blood smear of similarly amplified polyclonal DNA. For detection of monoclonality the present use of PCR is approximately as sensitive as Southern blotting, and both techniques are equivalent in being able to detect approximately 2% to 5% of a monoclonal population admixed in a larger polyclonal population. Therefore, they both provide a small advantage over morphology for detection of neoplastic cells. One real disadvantage of the PCR technique is the fact that monoclonality is not detected in all cases of B-lymphoproliferative disease. Southern blotting is superior in this regard, and for this reason our present approach to detection of monoclonality in lymphoproliferative disease is to first use PCR and reserve Southern blotting for those cases in which the PCR is negative.

Owing to its advantages, detection of monoclonality at the DNA level by use of PCR is likely to become a routine technique in the initial diagnosis of lymphoproliferative disorders. Its utility will be enhanced if an analogous method to detect T-lymphocyte monoclonality can be developed and if the phenomenon of false negativity can be overcome.

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