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Heteroduplex PCR analysis of rearranged T cell receptor genes for clonality assessment in suspect T cell proliferations

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Molecular analysis of T cell receptor (TCR) genes is frequently used to prove or exclude clonality and thereby support the diagnosis of suspect T cell proliferations. PCR techniques are more and more being used for molecular clonality studies. The main disadvantage of the PCR-based detection of clonal TCR gene rearrangements, is the risk of false-positive results due to 'background' amplification of similar rearrangements in polyclonal reactive T lymphocytes. Therefore, PCR-based clonality assessment should include analyses that discern between PCR products derived from monoclonal and polyclonal cell populations. One such method is heteroduplex analysis, in which homo- and heteroduplexes resulting from denaturation (at 94°C) and renaturation (at lower temperatures) of PCR products, are separated in non-denaturing polyacrylamide gels based on their conformation. After denaturation/renaturation, PCR products of clonally rearranged TCR genes give rise to homoduplexes, whereas in case of polyclonal cells heteroduplexes with heterogeneous junctions are formed. We studied heteroduplex PCR analysis of TCR gene rearrangements with respect to the time and temperature of renaturation and the size of the PCR products. Variation in time did not have much influence, but higher renaturation temperatures (>30°C) clearly showed better duplex formation. Nevertheless, distinction between monoclonal and polyclonal samples was found to be more reliable at a renaturation temperature of 4°C, using relatively short PCR products. To determine the sensitivity of heteroduplex analysis with renaturation at 4°C, (c)DNA of T cell malignancies with proven clonal rearrangements was serially diluted in (c)DNA of polyclonal mononuclear peripheral blood cells and amplified using V and C primers (*TCRB* genes) or V and J primers (*TCRG* and *TCRD* genes). Clonal *TCRB* and *TCRD* gene rearrangements could be detected with a sensitivity of at least 5%, whereas the sensitivity for *TCRG* genes was somewhat lower (10–15%). The latter could be improved by use of Vγ member primers instead of Vγ family primers. We conclude from our results that heteroduplex PCR analysis of TCR gene rearrangements is a simple, rapid and cheap alternative to Southern blot analysis for detection of clonally rearranged TCR genes.

Keywords: heteroduplex analysis; PCR; clonality assessment; T cell receptor (TCR); TCR genes; T cell malignancies

Introduction

Acute lymphoblastic leukemias can easily be diagnosed by routine cytomorphologic examination and/or by immunophenotyping. The latter is based on homogeneous expression of particular atypical leukemia-specific marker combinations, including the expression of terminal deoxynucleotidyl transferase (TdT).[1–3] Immunophenotypic diagnosis of the more mature leukemias and lymphomas is generally more complex. In case of mature B cell proliferations, single immunoglobulin (Ig) light chain (ie Igκ or Igλ) expression as determined by immunophenotyping, is indicative of the presence of a clonal B cell population.[4,5] However, clonality of suspect mature (TdT-negative) T cell proliferations is difficult to establish by immunophenotyping, because of the absence of tumor-specific markers. As lymphoid leukemias and lymphomas can be considered as malignant counterparts of normal T cells in their various differentiation stages,[6,7] most T cell malignancies contain rearranged T cell receptor (TCR) genes. Similar to other neoplasms, T cell malignancies are derived from a single malignantly transformed cell, implying that the TCR gene rearrangements within all the malignant cells are identical. Molecular analysis of TCR genes is therefore generally performed to prove or exclude clonality.[8–12] So far, this mainly concerned Southern blot analysis, which is a reliable method, if proper probe/restriction enzyme combinations are used.[10,11] Because Southern blot analysis of TCR genes is time-consuming and labor-intensive, PCR techniques are frequently being used as alternatives.

Although PCR techniques can be sensitive and fast, there are two major drawbacks in diagnostic clonality studies of TCR genes. Firstly, the possibility of identifying clonal rearrangements by PCR is limited by the choice of primers. Incomplete V-D or D-J rearrangements (in case of *TCRB* and *TCRD* genes) will remain undetected if only V and J primers are used. Furthermore, rearrangements involving particular V gene segments (for the *TCRB* genes) might remain undetected when only consensus primers are used. The second more important drawback is the risk of false-positive results due to 'background' amplification of similar rearrangements in polyclonal, reactive T lymphocytes. The latter implies that PCR analysis is not sufficient for clonality assessment, unless it is followed by analyses that discern between PCR products derived from polyclonal and monoclonal T cell populations. Methods that have been applied to solve this PCR 'background problem' include: direct sequencing of the PCR products,[13,14] single-strand conformation polymorphism (SSCP)-based analysis,[15,16] denaturing gradient gel electrophoresis (DGGE),[17–20] heteroduplex analysis,[21–23] temperature gradient gel electrophoresis (TGGE),[24] and gene scanning analysis.[25–28]

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From these techniques, heteroduplex analysis is probably the simplest, fastest and cheapest method for analysis of PCR products of rearranged TCR genes.

In heteroduplex analysis, PCR products are denatured at high temperature and subsequently renatured to induce homo- or heteroduplex formation. Originally the heteroduplex technique was designed for mutation detection in genetic diseases. However, it has been shown in several reports that it can also be applied for analysis of PCR products from TCR gene rearrangements.[21–23] Usage of heteroduplex analysis enables discrimination between PCR products derived from monoclonal and polyclonal lymphoid cell populations, based on the presence of homoduplexes (PCR products with identical junctional regions) or a smear of heteroduplexes (derived from PCR products with heterogeneous junctional regions), respectively (Figure 1). In this report heteroduplex analysis of rearranged TCR genes is studied with respect to time and temperature of renaturation and the size of the PCR products in order to further optimize the applicability of this technique and to determine its sensitivity for clonality assessment in suspect T cell proliferations.

Materials and methods

Cell samples

Cell samples were obtained from several TCR$\gamma\delta^+$ T cell acute lymphoblastic leukemias (T-ALL) and TCR$\alpha\beta^+$ T cell large granular lymphocyte (T-LGL) proliferations, containing $>$90% of malignant cells at initial diagnosis. Clonality of the T cells was proven with Southern blot analysis of TCR genes.[10] Mononuclear cells (MNC) were isolated from peripheral blood (PB) or bone marrow (BM) by Ficoll–Paque (density: 1.077 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation. The cell samples were frozen and stored in liquid nitrogen.

DNA isolation, RNA isolation and reverse transcriptase (RT) reaction

DNA was isolated from frozen MNC as described previously.[10] Total RNA was isolated essentially according to the method of Chomczynski.[29] cDNA was prepared from mRNA as described before,[30] using oligo(dT) and AMV reverse transcriptase.

(RT)-PCR amplification

PCR was essentially performed as described previously.[31] In each 100 μl PCR reaction 0.1–1 μg DNA sample, 12.5 pmol of 5′ and 3′ oligonucleotide primers, and 1 U *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA) were used. The oligonucleotides used for amplification of *TCRG* and *TCRD* gene rearrangements were published before.[12] For *TCRB* RT-PCR amplification cDNA (5 μl out of 20 μl RT reaction volume) was amplified in 100 μl reaction mixtures, containing 12.5 pmol of 5′ and 3′ primers and 1 U *Taq* polymerase. An RT-PCR procedure with a single Cβ primer in combination with 30 Vβ family-specific framework 3 (FR3) primers was set up, in order to avoid differences in primer annealing to the members of the 25 well-defined Vβ families when using a consensus Vβ primer.[32] The Vβ family-specific primers as well as the

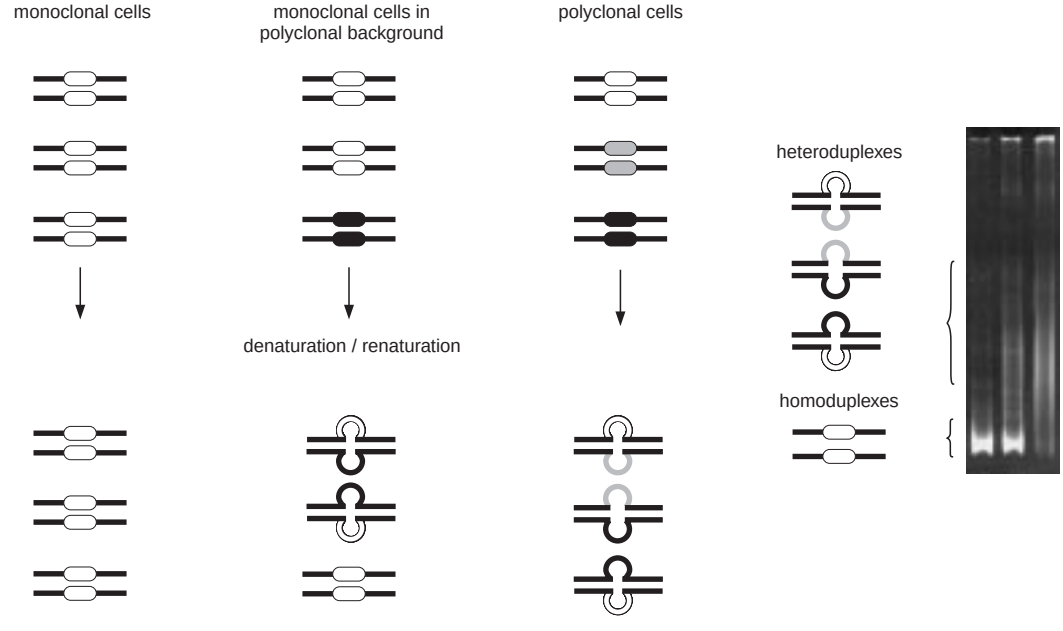

Figure 1 Schematic diagram of the heteroduplex analysis technique. In order to discern between PCR products derived from monoclonal and polyclonal lymphoid cell populations, the junctional region heterogeneity of PCR products of rearranged Ig or TCR genes can be studied by heteroduplex analysis. In heteroduplex analysis PCR products are heat-denatured and subsequently rapidly cooled to induce duplex (homo- or heteroduplex) formation. In cell samples which consist of clonal lymphoid cells, the PCR products of rearranged Ig or TCR genes give rise to homoduplexes after denaturation and renaturation, whereas in samples which contain polyclonal lymphoid cell populations the single-strand PCR fragments will mainly form heteroduplexes upon renaturation (left part). In case of admixture of monoclonal cells in a polyclonal background, both hetero- and homoduplexes are formed. Because of differences in conformation, homo- and heteroduplexes can be separated from each other by electrophoresis in non-denaturing polyacrylamide gels. Homoduplexes with perfectly matching junctional regions migrate more rapidly through the gel than heteroduplex molecules with less perfectly matching junctional regions. The latter form a background smear of slower migrating fragments (right part).

Cβ primer used for this *TCRB* RT-PCR were adapted from Gorski *et al*.[33] All oligonucleotides were synthesized on an ABI 392 DNA synthesizer (Applied Biosystems, Foster City, CA, USA) using the solid-phase phosphotriester method. PCR conditions were 1 min at 94°C, 1 min at 55° to 65°C, and 2 min at 72°C for 30 to 40 cycles using a Perkin-Elmer thermal cycler (Perkin-Elmer Cetus). After the last cycle an additional step of 7 min at 72°C was performed for final extension. RT-PCR conditions were 1 min at 94°C, 1 min at 65°C, and 2 min at 72°C for 35 cycles, also followed by a final extension step (7 min, 72°C).

Heteroduplex analysis

For heteroduplex analysis, the PCR or RT-PCR products were denatured at 94°C for 5 min, after the final cycle of amplification, and subsequently cooled (to a lower temperature) to induce duplex formation. This renaturation step was performed at different temperatures (range 4–40°C) for different time periods (range 15–60 min). After duplex formation the hetero- and/or homoduplexes were immediately loaded on 6% non-denaturing polyacrylamide gels in 0.5 × Tris-Boric acid-EDTA (TBE) buffer, run at room temperature, and visualized by ethidium bromide staining. *Pst*I-digested lambda DNA was used as a size marker.

Results

Influence of renaturation time and temperature on duplex formation

To find the optimal conditions for renaturation in heteroduplex analysis, the influence of both time and temperature of renaturation was studied for PCR products of rearranged TCR genes. For this purpose we used DNA from a TCR$\gamma\delta^+$ T-ALL patient with biallelic Vγ8-Jγ2.3 rearrangements. As both *TCRG* gene rearrangements in this patient involve the same gene segments, PCR amplification with VγI and Jγ1.3/2.3 primers[12] resulted in almost identical PCR products of around 450 bp. The two products only differed in their junctional regions. The heterogeneity was characterized by a 13 bp size difference and a difference in nucleotide composition. Upon denaturation and renaturation of these PCR products, four double-strand fragments were formed. These included two homoduplexes, representing the two PCR amplified rearranged alleles, and two distinct heteroduplex molecules resulting from renaturation of single-strand fragments of the PCR products from the two different alleles (Figure 2).

Following PCR amplification with VγI and Jγ1.3/2.3 specific primers, heteroduplex analysis was performed at variable renaturation temperatures to compare the degree of duplex formation on polyacrylamide gels. When renaturation was performed for 1 h at relatively high temperatures (30°C or 40°C) the two homoduplexes and the two slower migrating heteroduplexes were clearly detectable (Figure 2). After renaturation at room temperature (22°C), duplex formation was not complete, as slow-migrating single-strand products were also visible. At renaturation temperatures of 10°C or 4°C, single-strand fragments were even more clearly present in parallel with a lower intensity of the duplex band (Figure 2). This is probably due to the slower renaturation process at these lower temperatures. Analysis of biallelic Vγ8-Jγ2.3 rearrangements (11 nucleotides size difference) from a second TCR$\gamma\delta^+$ T-ALL patient revealed a similar pattern of duplex bands and single-strand products. Analysis of biallelic Vδ1-Jδ1 PCR products (around 450 bp, with a size difference of 33 nucleotides) in the latter patient sample also showed a comparable effect of temperature on duplex formation. Interestingly, in all cases the single-strand fragments showed different migration patterns at the different renaturation temperatures, suggesting that the conformation of single-strand DNA products is also temperature-dependent.

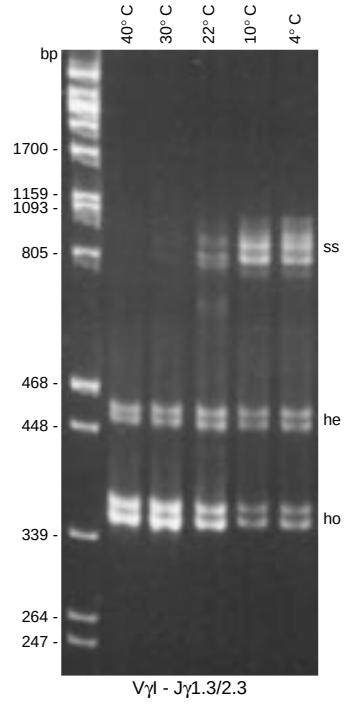

Figure 2 Analysis of the influence of the rearrangement temperature on duplex formation of (biallelic) *TCRG* PCR products from T-ALL DNA. After amplification of the clonal *TCRG* rearrangements with VγI and Jγ1.3/2.3 primers, PCR products were denatured for 5 min at 94°C and renatured for 1 h at the indicated renaturation temperatures. Electrophoresis was performed in 6% non-denaturing polyacrylamide gels, using *Pst*I-digested lambda DNA as size marker. As a result two homo- and two heteroduplexes can be identified. At higher temperatures (>30°C) the renaturation process is (almost) complete, whereas upon renaturation at lower temperatures single-strand fragments can still be identified as more retarded fragments. ss, single-strand fragments; he, heteroduplexes (of clonal origin); ho, homoduplexes.

To test the effect of renaturation time on duplex formation, several different *TCRG* and *TCRD* gene rearrangements were amplified and subsequently denatured and renatured for different time periods (15–60 min). However, in contrast to the differences observed upon variation in renaturation temperature, differences in time of renaturation (at a given temperature) did not have much effect on duplex formation (data not shown). Irrespective of the type of PCR product and the renaturation temperatures, maximum levels of homo- and heteroduplexes were generally observed after 30 to 45 min of renaturation. Duplex formation was only slightly lower after 15 min of reannealing. This illustrates that the degree of duplex formation is largely dependent on renaturation temperature rather than duration of the renaturation process. To guarantee optimal renaturation, we used renaturation times of 1 h in all further experiments.

Influence of renaturation temperature and size of the PCR product on clonality assessment

Although the above-mentioned results clearly indicate that duplex formation is more optimal at higher renaturation temperatures, we wanted to know whether the discussed denaturation/renaturation strategy can effectively be applied to discern between monoclonal and polyclonal cell populations. For this we took 100%, 20% and 0% dilutions of T-ALL DNA in polyclonal MNC DNA, PCR amplified the Dδ2-Jδ1 rearrangement, and subsequently performed heteroduplex analysis with renaturation at either 40°C or 4°C for 1 h. After renaturation the samples were kept on ice until loading of the gel, using ice-cold loading buffer. The polyacrylamide gels were run at room temperature.

The Dδ2-Jδ1 rearrangement was amplified using a Jδ1 primer in combination with either one of two distinct Dδ2 primers. The proximal primer, Dδ2, is located relatively close to the Dδ2 gene segment, resulting in a PCR product of around 240 nucleotides, whereas the distal primer (Dδ2up) lies far more upstream of the Dδ2 gene segment (and even upstream of the Dδ1 gene segment), giving rise to a much larger PCR product of around 800 bp. These two different Dδ2 primers were employed to see whether the size of the PCR product might be an important determinant for reliable clonality assessment as well. Amplification with the Dδ2up and Jδ1 primers, followed by denaturation and renaturation at 40°C, resulted in clear homoduplex bands of the expected size in the 100% and 20% leukemic samples, but a band of comparable size was also observed in the healthy control MNC sample (Figure 3a). When renaturation was performed at 4°C, the intensity of the homoduplex bands in the 100% and 20% samples was slightly lower (Figure 3a), as was expected from the above-described temperature experiments. Most importantly however, no homoduplexes were found in the control sample at this low renaturation temperature. The problem of detecting a 'false-positive' homoduplex band in the non-leukemic control sample could also largely be resolved when the more proximal Dδ2 primer was used for Dδ2-Jδ1 amplification (Figure 3b). Nevertheless, using this smaller PCR product, a faint homoduplex band could still be detected within a background smear in the polyclonal control sample. The 'false-positive' homoduplex band again entirely disappeared when renaturation was performed at 4°C (Figure 3b). Similar results were obtained with Vβ-Cβ RT-PCR products of even smaller size (around 200 bp), where renaturation at 40°C resulted in faint homoduplex bands that disappeared when reannealing was performed at 4°C (data not shown). These experiments showed that larger PCR products gave rise to stronger false-positive homoduplex-like bands at 40°C renaturation, whereas renaturation at 4°C never gave false-positive results.

Sensitivity of heteroduplex analysis for clonality detection

The above data suggested that a reliable distinction between monoclonal and polyclonal samples by heteroduplex PCR analysis requires renaturation at a low temperature (4°C). However, the initial experiments clearly indicated that duplex formation is less optimal at lower temperatures, suggesting that the sensitivity of the heteroduplex technique would be lowered by performing renaturation at 4°C. We therefore performed dilution experiments for several targets to study the sensitivity of detection of clonal homoduplexes upon renaturation at 4°C.

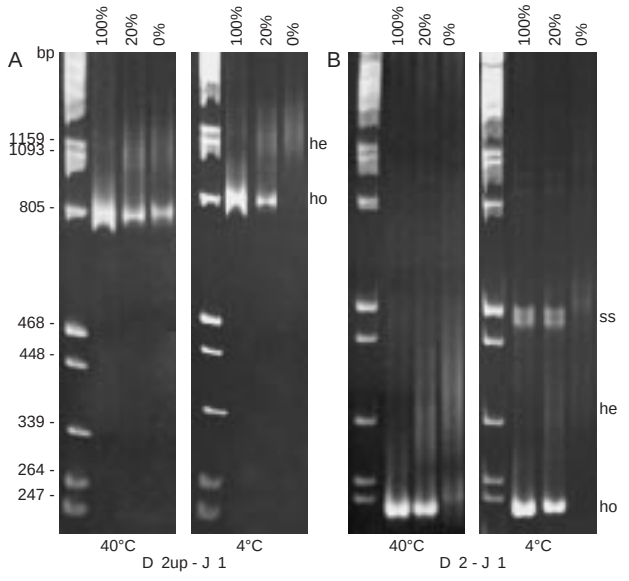

Figure 3 Clonality assessment via heteroduplex analysis of TCR junctional regions. T-ALL DNA was serially diluted (100%, 20%, and 0%) in polyclonal MNC DNA. The clonal *TCRD* rearranged gene was subsequently PCR amplified using either Dδ2up and Jδ1 primers (a) or Dδ2 and Jδ1 primers (b). The resulting PCR products were subjected to heteroduplex analysis; denaturation (5 min, 94°C) was followed by renaturation for 1 h at 40°C or 4°C. Samples were run in 6% non-denaturing polyacrylamide gels. *Pst*I-digested lambda DNA was used as size marker. In case of the large PCR products (a) a homoduplex-like band was visible in the polyclonal control sample after renaturation at 40°C, but not at 4°C. Although less clearly, this band was also visible upon renaturation of smaller PCR products (b) at 40°C, but again disappeared upon renaturation at 4°C. The single-strand fragments as visible in b (4°C) are not visible in panel a due to the slow mobility of the larger single-strand fragments. ss, single-strand fragments; he, (smear of) heteroduplexes; ho, homoduplexes.

Firstly, sensitivity of clonality detection was determined for several frequently occurring *TCRG* and *TCRD* gene rearrangements. Heteroduplex analysis of Vδ1-Jδ1 and Dδ2-Jδ1 amplified products, using serial dilutions of several TCRγδ$^+$ T-ALL, yielded clear homoduplex bands in samples containing only 5% tumor cells (Figure 4a and b). Occasionally faint homoduplex bands were even observed in 1% dilutions. Homoduplexes were never found when using polyclonal DNA. VγII(Vγ9)-Jγ1.3/2.3 homoduplexes could also be detected with a sensitivity of around 5% (Figure 5b). However, clonality detection of Vγ-Jγ1.3/2.3 rearrangements by heteroduplex analysis turned out to be more difficult, resulting in a sensitivity of around 10% (Figure 5a). This limited sensitivity can probably be explained by the fact that all rearrangements involving members of the Vγ family are amplified when using a Vγ family-specific oligonucleotide primer. We therefore determined the detection limit using Vγ member-specific primers to amplify *TCRG* rearrangements. This resulted in improved detection of homoduplexes with a sensitivity of at least 5%, which is at least two-fold better than in the case of the consensus Vγ family primer (Figure 5a).

PCR analysis of *TCRG* and *TCRD* genes can easily be performed at the DNA level because of the relatively limited number of V, (D), and J segments. As the high number of Vβ and Jβ gene segments would require many different primer combinations, analysis of *TCRB* genes is often performed via

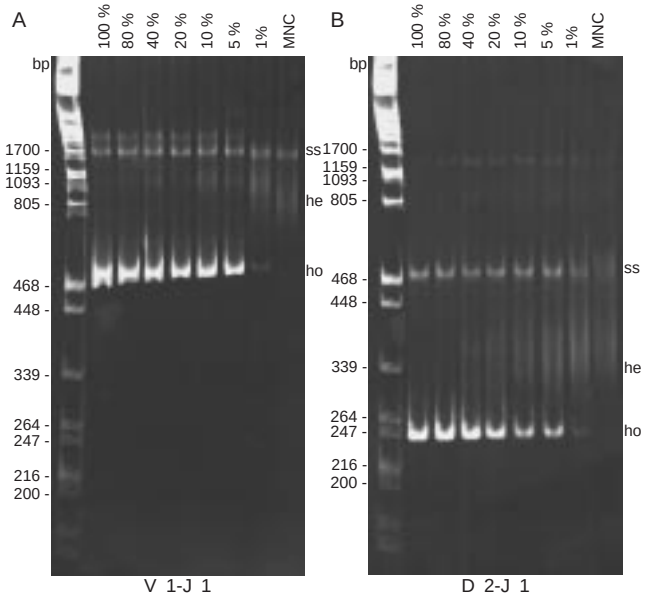

Figure 4 Sensitivity of heteroduplex analysis for clonality detection of *TCRD* rearranged gene products. T-ALL DNA was serially diluted in polyclonal MNC DNA. The clonal Vδ1-Jδ1 and Dδ2-Jδ1 rearrangements in this DNA sample were amplifed with specific primer sets, ie either Vδ1 (a) or Dδ2 (b) primers in combination with a Jδ1 primer. The resulting PCR products were subjected to heteroduplex analysis (5 min, 94°C; 1 h 4°C) prior to electrophoresis in 6% non-denaturing polyacrylamide gels. In both panels clonal homoduplexes could be detected with a sensitivity of 1–5%. ss, single-strand fragments; he, (smear of) heteroduplexes; ho, homoduplexes.

RT-PCR with Vβ family-specific primers and a single Cβ primer. Using this RT-PCR procedure, *TCRB* analysis was performed on several T-LGL proliferations as well as TCR$\alpha\beta^+$ T-ALL. Subsequently, cDNA from clonal Vβ2, Vβ7, and Vβ23 positive lymphoproliferations was serially diluted in polyclonal MNC cDNA and the PCR products were subjected to heteroduplex analysis with renaturation at 4°C. Homoduplexes were clearly visible in the 5% dilutions, whereas in the 1% dilutions a faint homoduplex could still be detected (Figure 6). In the polyclonal cDNA lane only a smear of heteroduplexes was seen.

Discussion

Southern blot analysis has long been the only reliable method for clonality assessment in suspect lymphoproliferations, but over the last years several PCR-based methods have been devised as an alternative. One of the main difficulties of PCR-based clonality detection is discrimination between monoclonal and polyclonal PCR products. This problem can be solved by further analyzing the PCR products in various ways, ranging from high resolution polyacrylamide gel electrophoresis (fingerprinting) and gene scanning[25–28] to DGGE[17–20] and heteroduplex analysis.[21–23]

In heteroduplex analysis hetero- and homoduplexes, resulting from denaturation and renaturation of PCR products, are separated in non-denaturing polyacrylamide gels based on their conformation. The presence of clear homoduplex bands or a smear of heteroduplexes enables discrimination between monoclonality and polyclonality, respectively (Figure 1). Therefore, heteroduplex analysis seems to be a simple, rapid and cheap technique of PCR product analysis, which can be introduced easily in most diagnostic laboratories, as no radioactive substrates are involved and no expensive equipment other than ordinary laboratory tools is required.

The number of clonal homoduplexes that is formed upon renaturation of denatured PCR products is related to the percentage of clonal T cells in the analyzed sample (Figure 7). However, based on the assumption that random pairing occurs between single-strand fragments of clonal and polyclonal T lymphocytes, the presence of polyclonal T cells in the same cell sample will lead to the formation of heteroduplexes. The more polyclonal T cells are present in the sample, the less homoduplexes (derived from monoclonal T cells) will be formed (Figure 7). Therefore, the sensitivity of heteroduplex PCR analysis for identification of clonal T cell populations between polyclonal T cells is dependent on the detectability of (renatured) homoduplexes amongst heteroduplexes. To optimize the heteroduplex technique for clonality assessment in suspect T cell proliferations further, we studied several assay parameters, like the time and temperature of renaturation and the size of the analyzed PCR products.

The temperature at which the denatured fragments renature rather than the time used for renaturation appeared to be an important determinant in duplex formation. Duplex formation is more optimal at higher renaturation temperatures (40°C) but clonality analysis appears to be more reliable at lower temperatures (4°C), especially in case of small suspect cell populations. This is caused by the presence of a faint, somewhat fuzzy homoduplex band in polyclonal control samples at 40°C, which is absent when renaturation is performed at 4°C. We assume that the higher movement of molecules at higher temperatures (eg 40°C) supports the non-random formation of 'non-clonal' homoduplexes due to preferential pairing of 'matching' polyclonal single-strand molecules. As a result many non-clonal homoduplexes with Gaussian size distribution are present at this high temperature, causing the fuzzy homoduplex band in polyclonal control samples (Figures 3a and b). This problem of 'false-positive' homoduplex bands is more prominent in case of larger PCR products (>600 bp) than in case of smaller (<250 bp) PCR products, in which the contribution of the junctional region is relatively high. It is our assumption that for large PCR products, in which the contribution of the junctional region nucleotides to the entire size of the PCR product is relatively small, the resolution of the PAGE system was not sufficient to discern between homoduplexes of identical size derived from clonal cells and differently sized non-clonal homoduplexes derived from polyclonal cells. Furthermore, large heteroduplex molecules with a few non-matching nucleotides will differ only slightly from homoduplexes in their mobility rate, since differences in electrophoretic mobility between heteroduplexes and homoduplexes progressively decrease when the PCR products become larger.

Heteroduplex analysis of TCR genes is thus a reliable technique for clonality assessment in suspect T cell proliferations, provided that the appropriate conditions are used, ie PCR products of preferably 150–250 bp and renaturation at 4°C. Analysis of serial dilutions of tumor DNA in polyclonal control DNA, revealed sensitivities of 1–5% for PCR products of *TCRD* gene rearrangements and also for RT-PCR products of *TCRB* transcripts. In case of VγII(Vγ9)-Jγ1.3/2.3 rearrangements a sensitivity of 5% was achieved as well, but VγI-Jγ1.3/2.3 rearrangements showed sensitivities of only around 10%. This reduced sensitivity is probably caused by the high background of these rearrangements, as they frequently occur in normal TCR$\alpha\beta^+$ T cells (Figure 7). Use of Vγ member-specific primers instead of a VγI family-specific primer can help

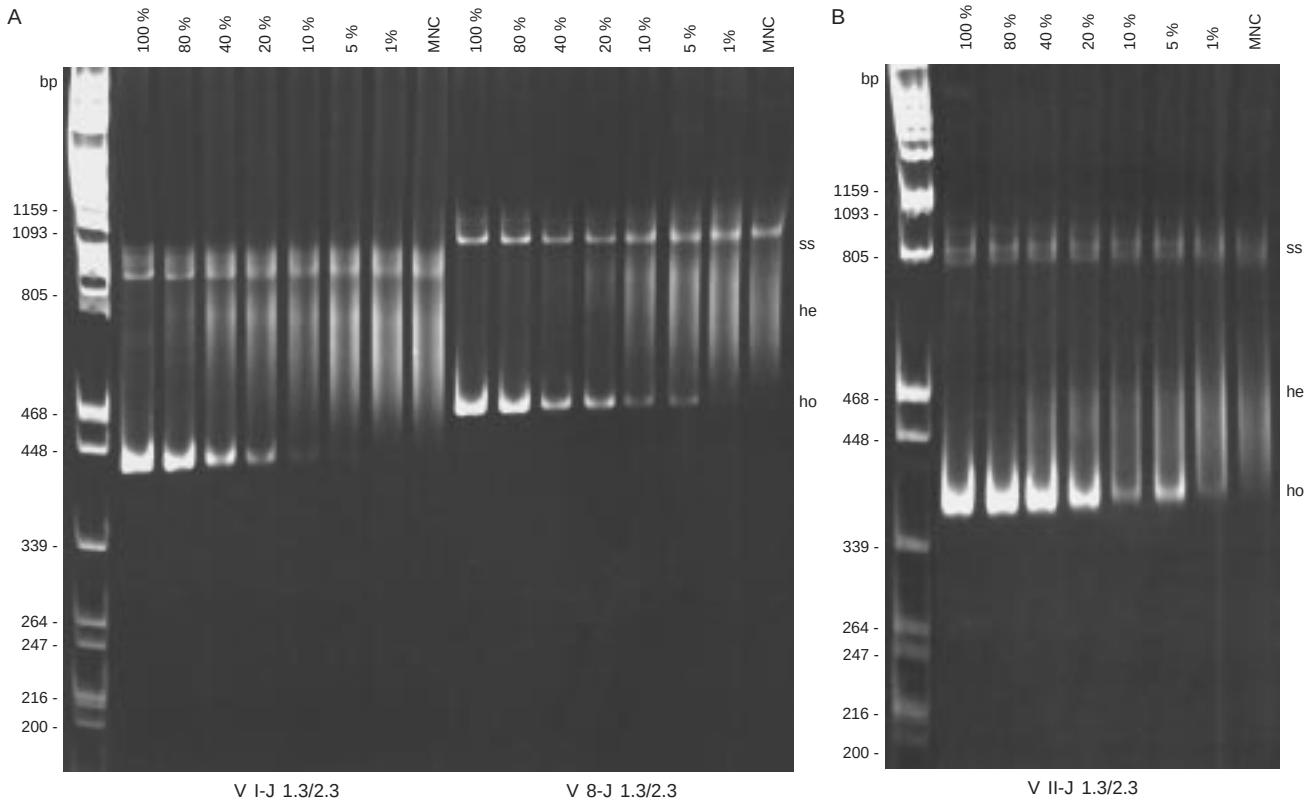

Figure 5 Sensitivity of heteroduplex analysis for clonality detection of *TCRG* rearranged gene products. T-ALL DNA was serially diluted in polyclonal MNC DNA. The clonal Vγ8-Jγ2.3 and Vγ9-Jγ2.3 rearrangements in this DNA sample were amplified with a family-specific V primer (Vγl) or a member-specific V primer (Vγ8) in combination with a Jγ1.3/2.3 primer (a) and a Vγll primer in combination with a Jγ1.3/2.3 primer (b). The resulting PCR products were subjected to heteroduplex analysis (5 min, 94°C; 1 h 4°C) before electrophoresis in 6% non-denaturing polyacrylamide gels. Clonal Vγll-Jγ2.3 homoduplexes could be detected with a sensivity of 1–5%. Sensitivity of detection of clonal Vγl-Jγ2.3 duplexes was similar, provided that member-specific Vγ primers were used. ss, single-strand fragments; he, (smear of) heteroduplexes; ho, homoduplexes.

to improve the sensitivity to at least 5% (Figure 5a). The less optimal duplex formation at 4°C could partly be compensated by prolonged renaturation (24 or even 48 h), without formation of false homoduplex bands in polyclonal control samples (data not shown). Increasing the renaturation time extensively may thus help to improve the sensitivity of heteroduplex analysis, which seems especially important for *TCRG* gene rearrangements. A detection limit of 1–5% for most TCR gene rearrangements is similar to or even better than Southern blot analysis. Moreover, the sensitivities that we reach under conditions that guarantee reliable clonality assessment, are largely comparable to those mentioned by other authors who used serial dilutions in polyclonal MNC DNA and performed high resolution non-denaturing PAGE[34] or DGGE.[17–20]

Sensitivities of around 5% are clinically relevant for initial diagnosis, but they are certainly not sufficient for detection of minimal residual disease (MRD) in ALL patients which requires sensitivities of 10^{-4} to 10^{-5}.[35] It has been suggested that sensitivities of 10^{-2} to 10^{-3} as determined by PCR might be predictive for slow remission upon chemotherapy,[36] but such sensitivities cannot easily be reached via heteroduplex analysis of PCR products, unless most of the polyclonal T cells are depleted before DNA or RNA extraction. Nevertheless, heteroduplex PCR analysis of TCR gene rearrangements is sufficiently sensitive for monitoring patients with chronic T cell leukemias as well as patients with oligoclonal T cell proliferations in order to predict the possible outgrowth of a dominant cell population.

Heteroduplex analysis of rearranged TCR gene products can be a valuable technique in several other applications as well. Proof or exclusion of the common clonal origin of two distinct lymphoproliferations in one patient can be obtained upon analysis of homo- and heteroduplexes after mixing PCR products of the two samples. Another application involves direct sequencing of homoduplex bands of PCR amplified rearranged TCR genes which obviates the need for cloning in order to sequence their junctional regions for MRD studies. Furthermore, heteroduplex analysis can also be employed for studying the diversity of the TCR repertoire in immune responses during infections or in autoimmune disorders, as has already been reported for the *TCRB* and *TCRG* genes.[21,22] However, this application suffers from a few technical limitations. Firstly, the heteroduplex method is more qualitative than quantitative; secondly, scarcity of material or low frequencies of T lymphocytes in the studied cell sample may give rise to a kind of pseudo-oligoclonality or even pseudo-monoclonality, which does not reflect the actual heterogeneity.

We conclude from our data that the described PCR procedure of TCR gene amplification followed by denaturation of the resulting PCR products and renaturation at 4°C serves to assess clonality in suspect T cell proliferations. This is especially the case when relatively short PCR products (150–250 bp) are analyzed. Heteroduplex analysis of PCR products is a simple, rapid and cheap alternative to Southern blot analysis for TCR gene clonality assessment. Moreover, hetero-

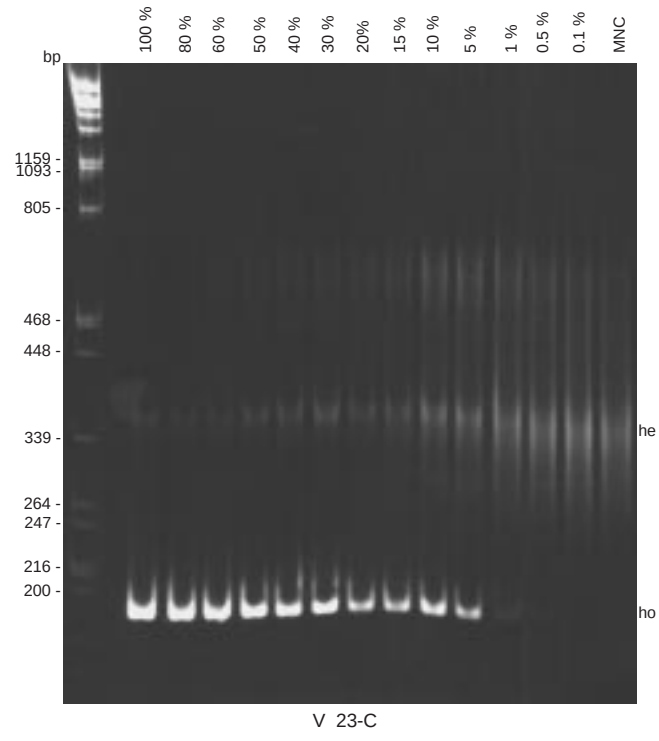

Figure 6 Sensitivity of heteroduplex analysis for clonality detection of *TCRB* rearranged gene products. T-LGL cDNA was serially diluted in polyclonal MNC cDNA. After reverse transcription, the clonal Vβ-Jβ rearrangement in this sample was amplified with specific primers, ie a family-specific V primer (Vβ23) in combination with a Cβ primer. The resulting PCR products were subjected to heteroduplex analysis (5 min, 94°C; 1 h 4°C) before electrophoresis in 6% non-denaturing polyacrylamide gels. Clonal homoduplexes could be detected with a sensitivity of 1–5%. ss, single-strand fragments; he, (smear of) heteroduplexes; ho, homoduplexes.

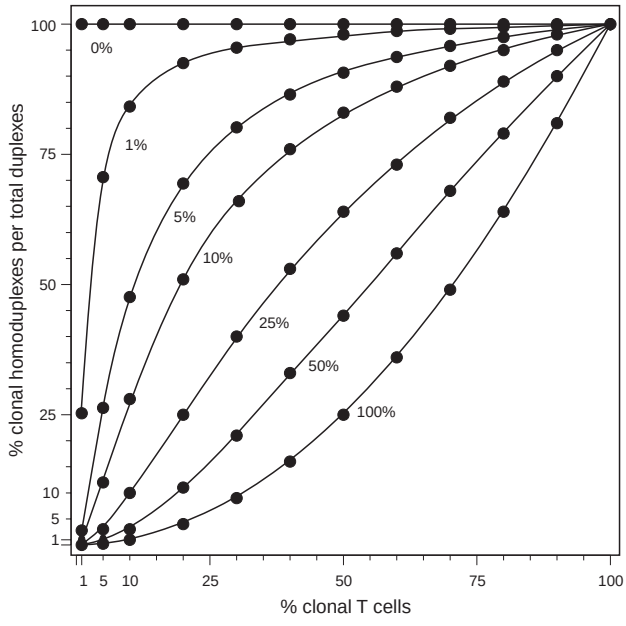

Figure 7 Calculated frequencies of clonal homoduplexes within the total pool of hetero- and homoduplexes, assuming that duplex formation is a fully random process as it seems to be at 4°C. The seven curves illustrate that the frequency of normal polyclonal T cells within the non-tumor cell fraction (0%, 1%, 5%, 10%, 25%, 50%, and 100%) drastically influences clonal homoduplex formation, since the frequency of clonal homoduplexes progressively decreases with the increase in the polyclonal T cell frequency. The percentage of clonal homoduplexes being formed by renaturation of single-strand fragments, was calculated according to the formula $H = M/(M + (100 - M).P/100) \times M/(M + (100 - M).P/100) \times 100\%$, in which H is the percentage of homoduplexes, M is the percentage of monoclonal T cells, and P is the percentage of polyclonal T cells in the non-tumor fraction.

duplex analysis may even be superior to other methods like fingerprinting, DGGE, and gene scanning, since no expensive equipment is needed, no radioactivity is involved, and different conditions do not seem to be required for all different TCR gene primer combinations. Our current results suggest that this procedure is not only useful for TCR gene analysis, but might also be applied for clonality asessment in suspect B cell proliferations via analysis of *IGH, IGK* and *IGL* genes. However, one should be aware that PCR studies of rearranged Ig genes in mature (post)follicular B cells might be hampered by the occurrence of somatic mutations.

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