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Evaluation of the Utility of Interphase Cytogenetics to Detect Residual Cells with a Malignant Genotype in Mixed Cell Populations: A Burkitt Lymphoma Model

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ABSTRACT

Interphase cytogenetics has been used to detect tumor cells in the presence of a large excess of normal cells. Probes for fluorescence *in situ* hybridization were chosen to reveal a specific hybridization pattern in tumor cell nuclei as well as to provide an internal control for the assessment of the hybridization results. By enumerating mixtures of cytogenetically normal cells and tumor cells from a Burkitt lymphoma cell line, we were able to detect tumor cells at a frequency of one in 500. Normal cells could be differentiated from Burkitt lymphoma cells with a specificity of ~99.9%.

INTRODUCTION

A CONSIDERABLE NUMBER of hematological malignancies exhibit specific and consistent chromosomal rearrangements (for review, see Heim and Mitelman, 1987). Progress in defining such chromosomal aberrations has been possible, to a large extent, due to the accessibility of mitotic peripheral blood cells to chromosome banding studies. Karyotype analysis, however, is restricted to metaphase chromosomes and, thus, depends on achieving a good mitotic index and high quality metaphase spreads. When only a limited number of chromosomal aberrant cells are present in a sample, karyotyping is not a suitable analytic technique.

Numerical chromosomal aberrations also can be detected in the interphase nucleus of a cell using nonisotopic *in situ* hybridization (Cremer *et al.*, 1986; Hopman *et al.*, 1988). Recent improvements of fluorescence *in situ* hybridization (FISH) protocols and the availability of an increasing number of suitable DNA probes allow the detection of even complex changes in chromosomal structure. Several publications have described the interphase detection of the *bcr-abl* fusion gene in chronic myelogenous leukemia (CML) (Arnoldus *et al.*, 1990; Tkachuk *et al.*, 1990), the disruption of the c-*myc* oncogene locus on 8q24 in Burkitt lymphoma (Ried *et al.*, 1992a), and the visualization of tumor-specific structural chromosomal aberrations involving the proto-oncogenes c-*fms*, c-*raf*-1, and c-*erbB*-2 (Lengauer *et al.*, 1992a). With the increasing availability of multiple reporter molecules and fluorochrome-based detector systems, as well as the introduction of nucleotide analogs directly conjugated to fluorochromes (Wiegant *et al.*, 1991), it now is possible to design sophisticated multicolor hybridization protocols (Nederlof *et al.*, 1990; Ried *et al.*, 1992a,b). The emergence of multicolor FISH technologies suggest that interphase cytogenetics holds considerable promise as a tool to detect residual pathological cells in a large background of cells with a normal karytype. To test the potential utility of interphase cell analysis for the enumeration of residual malignant cells, we chose to evaluate the t(2;8) translocation in a cell line of a female patient with Burkitt lymphoma. The hybridization probes were chosen to: (i) result in a specific hybridization pattern in tumor cells that is easily distinguishable from the hybridization pattern in normal cells, and (ii) provide an internal control for verifying the assessment of malignancy, independent of the breakpoint specific probe sets. This four-color FISH approach provides a highly specific method

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for the detection of cells bearing the t(2;8) translocation. The use of a charge coupled device (CCD) camera for the imaging of four fluorochromes is demonstrated and the practicability with respect to an automated image analysis is discussed.

MATERIALS AND METHODS

Cells and cell mixture

A cytogenetically normal male Epstein-Barr virus (EBV)-transformed cell line was kindly provided by Dr. Stephen Reeders (New Haven, CT) and metaphase spreads were prepared as described by Yunis (1976). The Burkitt lymphoma cell line JI carrying the t(2;8)(q32;q24) translocation was characterized earlier both by conventional G banding (Bornkamm *et al.*, 1980) and FISH (Ried *et al.*, 1992a).

Cells were cultured in RPMI containing 15% fetal calf serum. Prior to harvesting, the cell number was determined in a modified Neubauer counting chamber. Cells were given a hypotonic treatment (0.075 *M* KCl, 15 min, 37°C) and then fixed in methanol/acetic acid (3:1); the fixation was repeated five times. Cells from the normal control and from the Burkitt lymphoma cell line were mixed in ratios 10:1, 100:1, 500:1, and 1,000:1, and dropped onto clean wet slides. The slides were stored until use at −70°C in a light-tight box with drierite powder.

DNA probes

The X chromosome-specific centromeric repeat pXBR (Yang *et al.*, 1982) was prepared according to standard techniques (Sambrook *et al.*, 1989). Phage clones with inserts spanning the c-*myc* locus on 8q24 and the *pvt*-1 locus on 8q have been characterized previously (Henglein *et al.*, 1989). The phage clone L17, which maps to band 8q24.1, was kindly provided by H.-J. Lüdecke and Dr. B. Horsthemke (Essen, Germany). DNA was prepared following standard protocols (Silhavy *et al.*, 1984) and labeled by nick translation. Clone L17 and the three phage clones

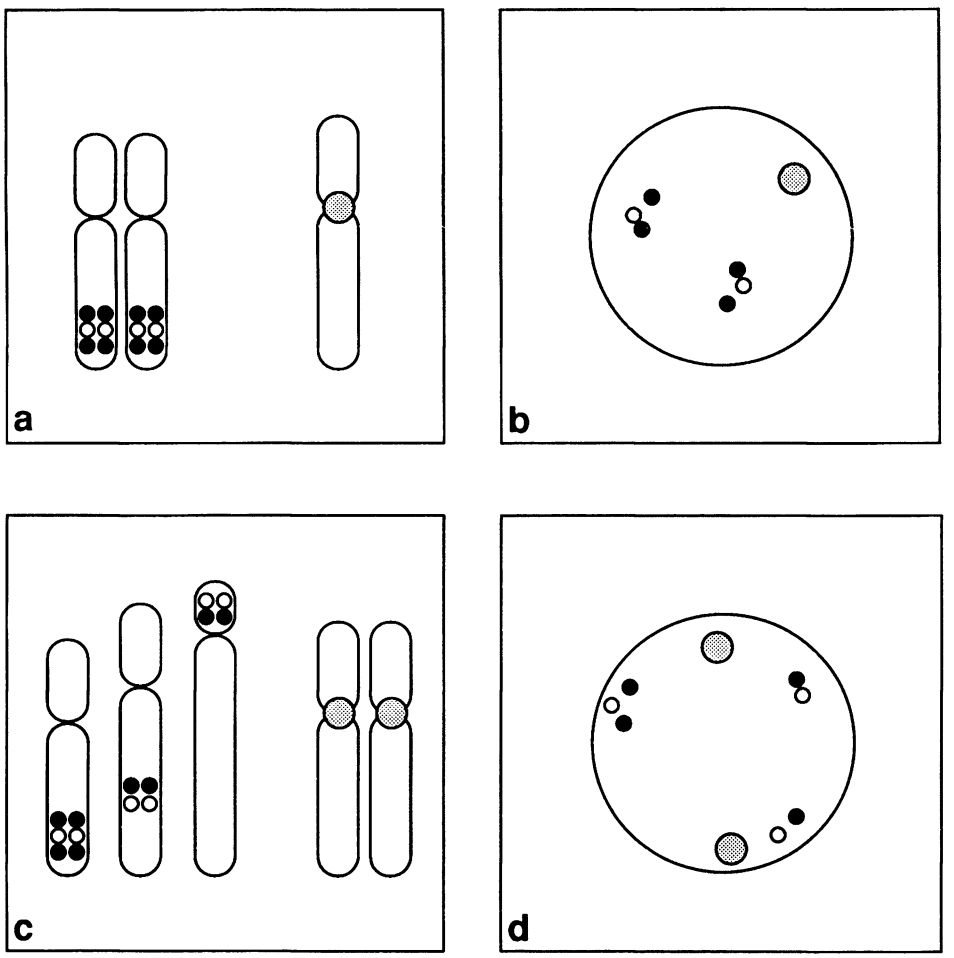
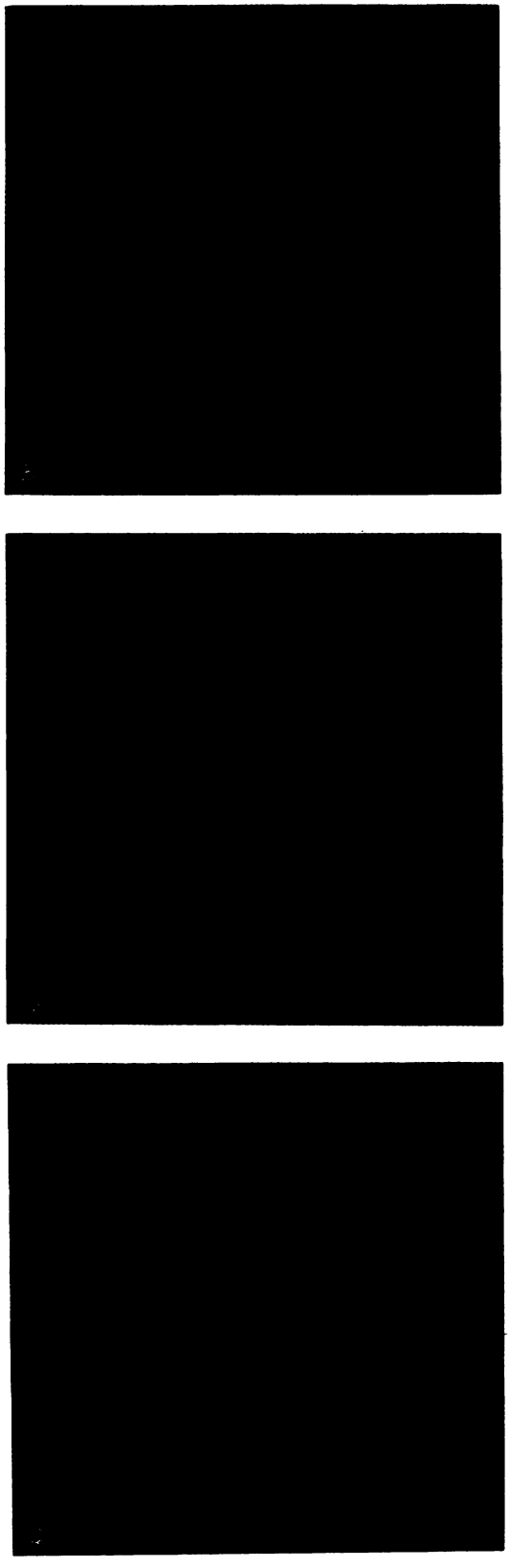


FIG. 1. Schematic presentation of the hybridization patterns on metaphase chromosomes and interphase nuclei. The phage clones spanning the translocation breakpoint (detected with FITC; green) are symbolized as blank dots. The flanking clones (detected with rhodamine; red) are presented as solid dots. The X centromere-specific signals (detected with Ultralite 680) are drawn as hatched dots (details see text). a and b. Expected hybridization pattern on a normal male metaphase spread and in a normal male interphase nucleus (b). c and d. Expected hybridization pattern on a metaphase spread (c) and in an interphase nucleus (d) from Burkitt lymphoma cells of a female carrying the t(2;8) translocation.



from the *pvt*-1 region were labeled with digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN). pXBR was labeled with biotin-11-dUTP (Sigma Chemicals, St. Louis, MO) and the nine phage clones spanning the t(2,8) translocation breakpoint were labeled with FITC-dUTP (Boehringer Mannheim).

In situ *hybridization*

Cells were pretreated as described elsewhere (Wiegant *et al.*, 1991). Briefly, slides were incubated with RNase A in 2× SSC (final concentration, 100 μg/ml) for 30 min at 37°C, followed by a digestion with pepsin (25 μg/ml final in 0.01 *M* HCl) for 10 min at 37°C and a postfixation step in 1% formaldehyde, 10 min, room temperature. Finally, slides were washed in 1× phosphate-buffered saline (PBS) and dehydrated through an ethanol series (70%, 90%, 100%).

For *in situ* hybridization of an 18 × 18-mm² area, 40 ng of phage L17, 60 ng of phage clones from the *pvt*-1 region (20 ng each), and 90 ng of phage clones (10 ng each) covering the breakpoint (see Ried *et al.*, 1992a), together with 10 μg of human competitor and 5 μg of salmon sperm DNA, were precipitated and resuspended in 5 μl of hybridization solution (50% formamide, 2× SSC). The DNA was heat denatured and allowed to preanneal for 2 hr at 37°C. DNA probe pXBR was precipitated in the presence of 5 μg of yeast tRNA, denatured separately in 5 μl of hybridization solution and combined with the phage DNA immediately before adding the probe cocktail to the slides. Two 18 × 18-mm² fields were hybridized per slide.

After overnight incubation, post-hybridization washes, and a blocking step (30 min, 37°C, 4× SSC, 3% bovine serum albumin), the digoxigenin-labeled probes were detected with anti-digoxigenin rhodamine (Boehringer Mannheim) and the pXBR signal was detected with streptavidin conjugated with the infrared dye Ultralite 680 (Ultra Diagnostic Corporation, Seattle, WA). Slides were counterstained with DAPI and embedded in DABCO (Sigma Chemicals) to reduce fading.

Evaluation of hybridization signals

Prior to *in situ* hybridization, the slides were coded. According to the hybridization pattern, cells were classified in the categories "normal," "tumor," or "indecisive" (see Re-

FIG. 2. Metaphase spreads and interphase nuclei after multicolor FISH. The green signals indicate the position of nine phage clones from the c-*myc* region in 8q24. The telomeric red signals reveal the position of three phage clones from the *pvt*-1 region. The red signals centromeric of c-*myc* display the position of phage clone L17. a. Partial normal male metaphase spread after hybridization with the probe set. The image was taken with an Agfa matrix procolor slide printer. b and c. A normal male nucleus (b) and a nucleus of the female Burkitt lymphoma cell line JI (c) after hybridization with the probe set. Photographs were taken directly from the computer screen using Kodak HC color slide film. The pink colors in band c indicate the X centromere.

sults for selection criteria and details). Subsequently, as an internal control, the infrared signals (which cannot be seen by eye) were imaged with a cooled CCD camera.

Digital imaging and photographs

Images were taken separately with specific filters for each fluorochrome, custom manufactured by Zeiss (Filter #487910 for fluorescein, #487915 for rhodamine, #487901 for DAPI). The spectral characteristics of the infrared filter were as follows: excitation 620–658 nm, dichroic 650 nm, bandpass 670–680 nm. Samples were analyzed using a Zeiss axioskop microscope equipped for epifluorescence using a cooled CCD camera (PM512, Photometrics, Tucson, AZ). The gray scale images were pseudocolored and merged using a software program developed in the laboratory of D.C.W. by Timothy Rand on a Apple McIntosh IIcx computer. This software package, termed "Gene Join," is available upon request through the Office of Cooperative Research (Yale University, 246 Church St, New Haven, CT 06510). Photographs were taken directly from the screen using a Kodak 100 HC color slide film (for details, see Ried *et al.*, 1992b).

RESULTS

To define the sensitivity and specificity of interphase cytogenetics for the detection of cells carrying structural chromosomal aberrations in an excess of normal cells, we chose the Burkitt lymphoma cell line JI which was established from a female patient and carries the translocation t(2;8)(q32;q24) (Bornkamm *et al.*, 1980). The control cell line has a normal male karyotype (46,XY). The chromosomal position of the phage clones used in this experimental setup are shown schematically in Fig. 1a. The open circles on chromosome 8 indicate the pool of nine phages that includes the c-*myc* gene in 8q24 (observed experimentally as green signals). The two black circles centromeric to the c-*myc* locus represent hybridization signals of the clone L17. The black circles telomeric to c-*myc* indicate the hybridization locus of the *pvt*-1 phage clones. Both of these loci are observed experimentally as red fluorescent spots. Figure 2a shows a metaphase spread after FISH of these phage clones to extended normal metaphase chromosomes.

The combined probe set described above generates a pattern of hybridization signals that can readily distinguish between normal cells and tumor cells of the interphase nuclei. These hybridization patterns are schematically presented in Fig. 1, b and d. In normal nuclei, we expect two green signals, each flanked by two red signals (Fig. 1b). In the Burkitt lymphoma cell line JI, carrying the translocation t(2;8), three green signals are expected. One green signal is derived from the normal chromosome 8 homolog and flanked by two red signals. The other two green spots indicate the splitting of the 8q24 c-*myc* region onto the two translocation chromosomes; each of these green signals, however, should be co-localized with only one red signal (Fig. 1d). In normal male cells, a single X-specific signal from the pXBR clone should be observed. Because the Burkitt lymphoma cell line JI is derived from a female patient, two X centromere-specific signals are expected. Enumeration of the number of X chromosome-specific signals provides an internal, yet independent, control on our ability to identify the malignant cell subpopulation correctly. Secure criteria to exclude background spots are particularly important if the enumeration is to be done in interphase nuclei. In contrast to analyses using metaphase chromosome preparations, background signals of similar size, shape, and color as the real signals are less easily recognized as background noise in interphase nuclei, because the outline shape of the metaphase chromosomes cannot be used to assess signal quality (*e.g.*, hybridization signals on both chromatids). To avoid counting of unspecific background signals and to exclude the enumeration of incomplete hybridization patterns, we defined three classification categories (normal, aberrant, or indecisive). A cell nucleus was regarded as normal only if the hybridization pattern was the same as the idealized one presented in Fig. 1b. Nuclei were assigned to the category "aberrant" only when the signal constellation after FISH resembled the pattern in Fig. 1d. All constellations revealing green signals not colocalized to red ones were placed in the indecisive category as were nuclei where the chromosome 8 homologs were tightly juxtaposed so that it was not possible to make an unambiguous characterization. After the cells were allocated to one of the three described categories, the filter was changed and the infrared fluorescence from the X centromere-specific probe was imaged. The cohybridization with the X centromere-specific probe was used to confirm whether a cell was male or female in origin and correctly judged as normal or aberrant; these determinations were always made following the visual inspection of the hybridization pattern of the chromosome 8 probe sets. Typical examples of a normal interphase nucleus (Fig. 2b) and a nucleus of the Burkitt lymphoma cell line JI (Fig. 2c) after four-color FISH analyses are shown in Fig. 2. Four mixtures of normal and aberrant cells (in ratios of 10:1, 100:1, 500:1, 1,000:1) were made and the number of normal and tumor cell nuclei enumerated. The results of the evaluation are shown in Fig. 3.

Although we omitted any immunological signal amplification step, 49% of the nuclei could be assigned to the categories "normal" or "aberrant" directly through the microscope; an additional 39% become scorable after imaging the signals with a CCD camera. The remaining 12% were summarized as "indecisive." The rates of indecisive hybridization patterns did not differ in any of the four cell mixtures (χ^2 test with 3 df, $p = 0.597$). To test the diagnostic value of the experimental model, we combined the data with all four mixtures and estimated the statistical parameters "sensitivity" and "specificity." Here, "sensitivity" refers to the proportion of correctly identified aberrant cells relative to the number of aberrant cells detected. "Specificity" indicates the proportion of cells showing a normal hybridization pattern among those cells defined as normal following the enumeration of X specific signals. The statistical evaluation is summarized in Table 1.

DISCUSSION

The obvious importance of detecting small numbers of malignant cells among an excess of normal cells has resulted in the introduction of several methods for the detection of these residual disease cells. Flow cytometry was used to screen for monoclonal B lymphocytes in non-Hodgkin lymphomas (Smith *et al.*, 1984). Southern blotting was used to detect aberrant genomic DNA fragments in B-cell lymphomas and acute lymphoblastic leukemia (Hu *et al.*, 1985). However, the sensitivity of these approaches is quite low (not exceeding 1–5%). The polymerase chain reaction (PCR) has proven to be considerably more sensitive in detecting minimal residual diseases because only a few template molecules are necessary for the amplification of specific DNA fragments. PCR was used, *e.g.*, for the detection of hybrid genomic DNA sequences in a t(8;14) translocation specific for follicular lymphoma (Lee *et al.*, 1988), and the detection of RNA sequences derived from the *bcr-abl* fusion gene in CML (Morgan *et al.*,

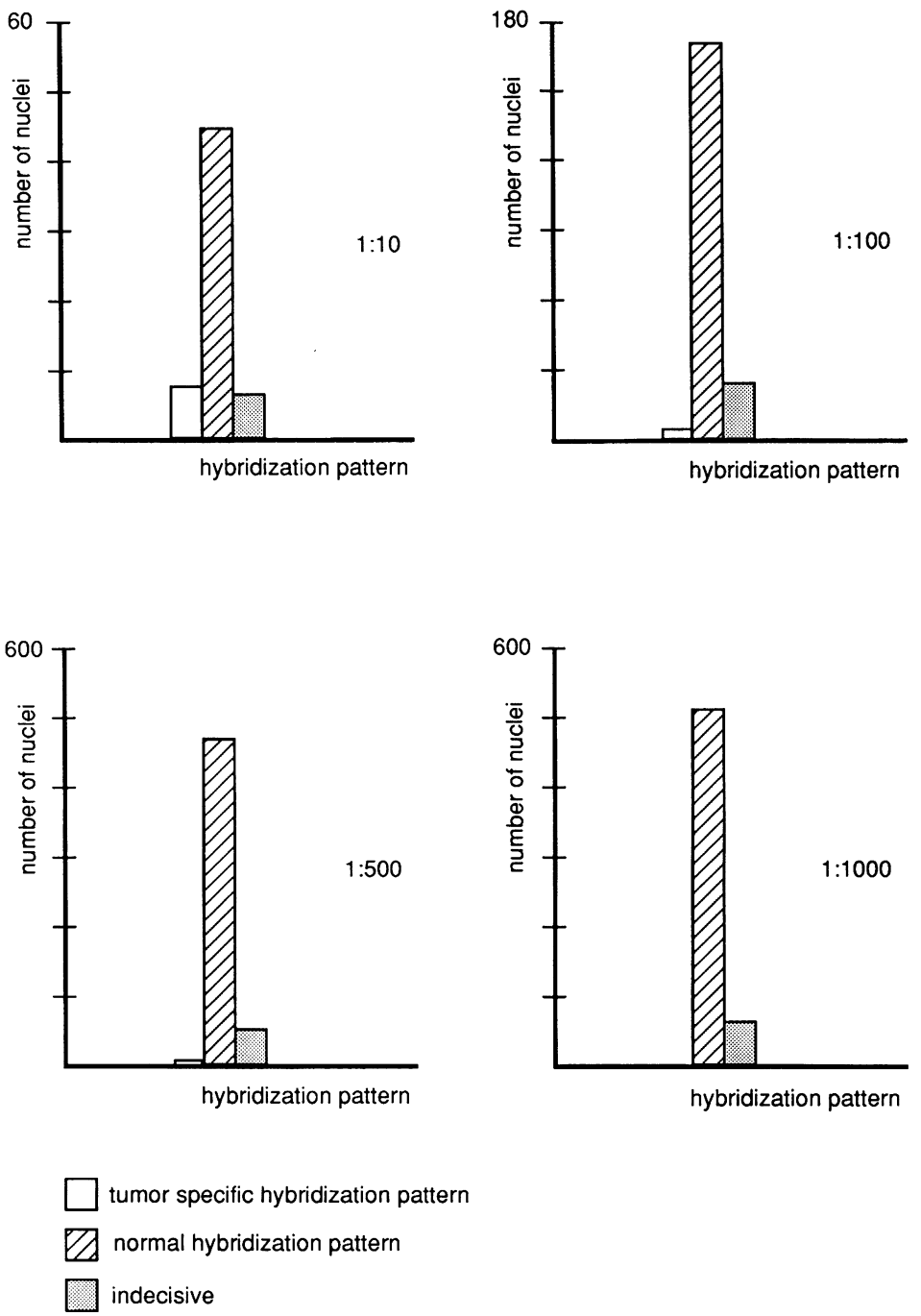


FIG. 3. Schematic presentation of the evaluation of the mixture preparations (1:10, 1:100, 1:500, 1:1,000).

TABLE 1.

	Correctly assessed	*Incorrectly assessed*	n	*Percent*	*Confidence interval*
Sensitivity	13	1	14	92.9	66.1-99.8
Specificity	1,332	1	1,333	99.9	99.6-99.9

n, Number of enumerated nuclei.

1989; Delfau *et al.*, 1990). The sensitivity for the detection of altered target sequences was 1:100,000. However, the presence of variant translocation sites or the absence of tumor-specific transcripts are two limiting factors for this technique. Contamination, nonspecific amplification, and the lack of reliable PCR quantification can create analytical problems as well.

FISH provides two major advantages for the analysis of residual disease cells: (i) the ability to investigate chromosomes throughout all stages of the cell cycle at the single cell level (Cremer *et al.*, 1986; Hopman *et al.*, 1988) and (ii) the ability to use multiple probes simultaneously (Nederlof *et al.*, 1990; Ried *et al.*, 1992b). However, because the evaluation of the hybridization patterns, especially in interphase nuclei, can sometimes be troublesome, it is imperative that strict criteria for signal evaluation are defined. Multicolor hybridization protocols (*e.g.*, Ried *et al.*, 1992a), using nested probes also permit better discrimination of real and artificial fluorescence spots, since only hybridization signals that show colocation of two (or more) different fluorescence signals will be scored as positives.

No detailed study on the sensitivity and specificity of FISH using locus-specific single gene probes has been performed so far. In this initial study, our estimation of test specificity appears to be very good. In only 0.01% of the evaluated cells (n = 1,332) false-negative results were obtained (*i.e.*, normal cells evaluated as tumor cells). Concerning the sensitivity issue, our estimation is rather poor, mainly due to the low number of aberrant cells present in the cell mixtures. Further experiments with increased levels of aberrant cells should improve the statistical evaluation (see also Ried *et al.*, 1992a). The number of nuclei comprising the "indecisive" category (12%) can be further reduced by using probes with higher hybridization efficiency. Recently, we have shown that *Alu*-PCR products generated from yeast artificial chromosomes (YAC) clones are extremely well suited for interphase cytogenetics since FISH signal efficiencies of almost 100% are obtained in methanol acetic acid-fixed peripheral blood lymphocytes (Lengauer *et al.*, 1992a,b). By combining YAC-clones specific for aberration prone regions with multicolor FISH protocols, it should be possible to develop sensitive diagnostic assays. The excellent hybridization properties of *Alu*-PCR-amplified YAC clones will also compensate for the lower detection efficiency of FISH signals on clinical specimen, *e.g.*, bone marrow smears (Bentz *et al.*, 1993). In addition, such multicolor FISH hybridization strategies could easily be coupled with automated image analysis devices. With automated "FISH cytometry" instrumentation, the analysis of a large cell population could be performed in a reasonable time. Rapid detection of low numbers of residual disease cells, particularly in hematopoietic cell populations, also suggests that interphase cytogenetics can be used for evaluating the effectiveness of chemotherapy regimens or the regular screening of "finger-prick" blood samples to monitor for possible onset of malignancy relapse.

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