Identification of Malignant Cells in Multiple Myeloma Bone Marrow with Immunoglobulin V_H Gene Probes by Fluorescent In Situ Hybridization and Flow Cytometry

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Abstract

Because it has been difficult to identify and separate malignant cells in human lymphoid malignancies, we have developed a flow cytometry–based fluorescent in situ hybridization (FISH) technique using immunoglobulin (Ig) heavy chain variable region (V_H) gene probes. After obtaining the specific V_H gene sequence expressed by the multiple myeloma IM-9 cell line and the malignant cells in five multiple myeloma patients, sense and antisense biotinylated single-stranded RNA probes were prepared by transcription from the malignant clone's V_H DNA sequences. The cells from the IM-9 cell line and from the mononuclear bone marrow cells of multiple myeloma patients were fixed, hybridized with the above biotinylated RNA probes, incubated with streptavidin-phycoerythrin, and analyzed by FACS® analysis. The myeloma cells stained positive with their own specific antisense V_H biotinylated RNA probes, whereas sense and irrelevant antisense biotinylated probes demonstrated only background staining. Dilutional concentrations of the IM-9 cell line with normal bone marrow cells were also accurately quantitated by this procedure. The application of this technique will allow a more accurate assessment of tumor burden in patients with multiple myeloma and should permit an accurate method of tumor cell purification for clinical as well as biological studies. Furthermore, this technological advance should be equally effective at identifying specific V_H gene–expressing cells in other lymphoid malignancies, as well as in nonmalignant B cell disorders. (*J. Clin. Invest.* 1995. 95:964–972.) Key words: complementarity determining region • antisense RNA probe • plasma cell • B cell separation • minimal residual disease

Introduction

The expression of genes in a clonal population of cells has been previously defined and quantitated by Northern blot and polymerase chain reaction (PCR) analysis (1–3). However, in heterogeneous cell samples in which only a fraction of cells express the gene of interest, these techniques are unable to precisely quantitate the percentage of cells with gene expression. To define gene expression of individual cells, in situ hybridization techniques have been developed using radioactive, chromatogenic, or fluorochrome-labeled agents. However, these methods must be performed on limited numbers of cells held on microscope slides. Flow cytometry–based fluorescent in situ hybridization (FISH)[1] methods using chromosome and/or gene markers have been developed to define specific karyotypic abnormalities in large populations of cells in suspension (4–7).

Multiple myeloma (MM) is a B cell malignancy resulting in the accumulation of a clonal population of plasma cells in the bone marrow which secrete a monoclonal immunoglobulin (Ig) protein. In our laboratory, we have sequenced the Ig heavy chain obtained from numerous patients with myeloma and have demonstrated that all members of the malignant clone in a given patient express the same heavy chain variable region (V_H) gene sequence (8, 9). Furthermore, this unique sequence does not vary with time over the course of the disease. Consequently, this gene may serve as a marker for identifying all malignant cells in this clonal B cell malignancy (8–10). In these malignant cells, the most specific region of the Ig gene sequence resides in the V_H segment. Since this V_H gene sequence is both specific and sensitive for the tumor cells in MM, we attempted to develop a FISH flow cytometric technique using biotinylated antisense probes to the expressed V_H gene as a means of identifying and separating malignant cells in this B cell tumor.

Methods

Myeloma cell line characteristics. IM-9, an IgG κ secreting human multiple myeloma cell line (American Type Culture Collection, Rockville, MD) (11), was grown in RPMI 1640 (Irvine Scientific, Santa Ana, CA) with 10% fetal bovine serum and supplemented with 2 mM L-glutamine.

Patient material. Bone marrow cells were obtained from five patients with IgG-producing MM and from one normal donor after informed consent and approval from our Human Subjects Protection Committee. The heparinized sample was layered over Histopaque 1077 (Sigma Immunochemicals, St. Louis, MO) centrifuged at 400 *g* for 30 min, and the mononuclear layer was obtained and washed three times in PBS after red blood cell (RBC) lysis with RBC lysis buffer (Sigma Immunochemicals).

V_H gene determination. Total RNA was extracted using the guanidine isothiocyanate method (12) from 30×10^6 IM-9 or myeloma bone marrow mononuclear cells. 50 pmol of an antisense primer specific for

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1. *Abbreviations used in this paper:* BM, bone marrow; CDR, complementarity determining region; DEPC, diethylpyrocarbonate; FC, flow cytometry; FISH, fluorescent in situ hybridization; HH, Hank's balanced salt and Hepes solution; MM, multiple myeloma; PE, phycoerythrin; T, Tween 20; V_H, heavy chain variable region.

Table I. Human Immunoglobulin V_H Gene family, Cγ, and Specific CDR2 Region Leader Primers Used for PCR Amplification

Primer name	Primer sequence
V_H1	5′ CCA TGG ACT GGA CCT GGA GG 3′
V_H2	5′ ATG GAC ATA CTT TGT TCC AC 3′
V_H3	5′ CCA TGG AGT TTG GGC TGA GC 3′
V_H4	5′ ATG AAA CAC CTG TGG TTC TT 3′
V_H5	5′ ATG GGG TCA ACC GCC ATC CT 3′
V_H6	5′ ATG TCT GTC TCC TTC CTC AT 3′
Cγ	5′ GCC CCC AGA GGT GCT CTT GGA GGA GG 3′
IM-9 CDR2 sense	5′ GGA GTG GGTCGC AGG TAT 3′
IM-9 CDR2 antisense	5′ AGT TGT CTC TGG AGA TGG 3′
WAD#35 CDR2 sense	5′ AGT GGG TCT CAA GTA TTA C 3′
WAD#35 CDR2 antisense	5′ TGT TGT TCT GGA TAT GGT G 3′

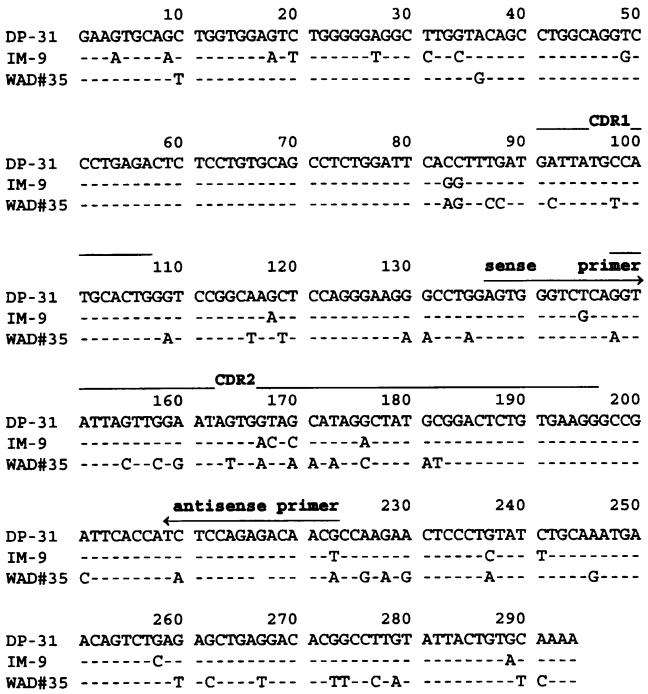

Figure 1. Sequence comparison of the IM-9 cell line and WAD#35's myeloma V_H genes with the most homologous germline gene for both which was V_H3 DP-31 (15) (*dashes* indicate identity, *underlines* indicate the CDR regions, and *arrows* indicate the primers used to amplify the short CDR2 region probe). Sequences were obtained after cDNA amplification of BM RNA using V_H family (13) and Cγ oligonucleotide probes.

Cγ (Table I) was hybridized to 1 μg of RNA by heating to 65°C and then cooling on ice. The Ig RNA was then reverse transcribed by using 200 U of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, MD) in a 30-μl volume of 1× first strand buffer at 42°C for 1 h after the addition of 0.66 mM of each deoxynucleotide triphosphate (Stratagene, La Jolla, CA), 40 U of RNase Block I (Stratagene), and 1 mM DTT (GIBCO BRL). After heat inactivation of the reverse transcriptase at 95°C for 5 min, the reaction was then cooled on ice. 50 pmol of a consensus primer to the leader sequence of one of the six V_H gene families (Table I) was added to the cDNA mixture along with 2.5 U *Taq* polymerase (Stratagene), and the sample volume was increased to 100 μl with 1× *Taq* polymerase buffer. Each PCR cycle consisted of 1 min of denaturation at 94°C, 1 min of annealing at 50°C, and 2 min of extension at 72°C, except for an initial 5 min at 94°C and a final 7 min of extension at 72°C. A total of 33 cycles of PCR amplification were performed. Six concurrent reactions were performed for each PCR amplification using a sense primer from each of the six V_H gene families (13). To visualize the PCR products, 10 μl of the PCR product was electrophoresed through a 1.5% agarose gel stained with ethidium bromide and examined under ultraviolet light. To purify the PCR product, the remainder of the PCR reaction was electrophoresed through a 1.5% low melt agarose gel, and the appropriately sized band was excised. A centrifuge filter unit (Spin-X; Costar Corp., Cambridge, MA) was used to extract the DNA from the agarose. The PCR fragment was then cloned directly into the SrfI site of the PCR-Script SK(+) vector according to the Stratagene instruction manual. Colonies containing appropriately sized PvuII (Stratagene) cut inserts were selected after Magic Miniprep (Promega Corp., Madison, WI) purification. Dideoxy chain termination sequencing was performed using the Sequenase II kit (United States Biochemicals Corp., Cleveland, OH).

To test whether a shorter RNA probe could improve assay specificity and efficiency, shorter PCR fragments were prepared for the IM-9 cell line and for MM patient WAD#35. Individual specific primers were synthesized (Operon Technologies, Inc., Alameda, CA) complementary to regions in the complementarity determining region 2 (CDR2), which were highly specific for the myeloma sequence after comparison with other germline V_H gene sequences (14, 15) (Fig. 1). 50 pmol of each primer was added to 1 μg of total RNA obtained from the IM-9 cell line or myeloma patient WAD#35 bone marrow cells, and the PCR product was synthesized using the procedure described above. A shorter 89-bp PCR product was obtained from these cells using the IM-9 cell CDR2 specific primers while a shorter 87-bp PCR product was made for MM patient WAD#35 using the patient CDR2 specific primers. These shorter PCR fragments were cloned into the Srf I cut PCR-Script vector using the above procedure. A plasmid containing the long or short PCR fragment would then be used as a template in the preparation of the RNA probe as outlined below.

RNA biotinylated probe preparation. All single-stranded RNA probes were prepared by transcription from the synthesized plasmid DNA. The plasmid DNA was linearized with either BamHI or NotI. Next, sense or antisense RNA transcripts were generated with either T3 or T7 DNA-dependent RNA polymerase using the Transcription *In Vitro* Systems kit (Promega Corp.). Biotin-labeled probes were prepared by substituting biotin-11 UTP (Enzo Biochem Inc., New York) or digoxigenin-11-UTP (Boehringer Mannheim Biochemicals, Indianapolis, IN) in the transcription reactions. Each probe had a near equivalent "U" content in its sense and antisense orientation. After performing the in vitro transcription reaction, RQ1 RNase-free DNase (Promega Corp.) was added to digest the DNA template for 15 min at 37°C. The probe was resuspended in diethylpyrocarbonate (DEPC)-treated water and was visualized after denaturing gel electrophoresis before purification by passage through a Sephadex G-50 Quick Spin Column for RNA (Boehringer Mannheim Biochemicals). The RNA probe concentration was determined by the method described by Ravichandran (5).

A 0.1-μg aliquot of the RNA sense and antisense probes was treated by adding 10 μg/ml RNase at 37°C for 15 min as a control experiment to determine whether an intact RNA probe was necessary for hybridization specificity. All of the probes were incubated with 1% DEPC at 37°C for an additional 15 min before use.

Cell fixation. The fixation and hybridization conditions were adapted from Bauman et al. (4). All solutions were made with DEPC-treated water, and all reagents and equipment were kept free of RNase. The cells were washed twice in a buffer containing 1× Hank's balanced salt solution and 20 mM Hepes, pH 7.2 (HH buffer). After washing, all further steps were carried out in 1.5-ml microfuge tubes. The cells were resuspended at 15×10^6 cells/0.5 ml HH and fixed by the addition of

an equal volume of freshly prepared 2% formalin in HH. After incubation for 5 min at room temperature, the cells were spun at 300 *g* for 5 min, washed once with HH, and resuspended in 0.3 ml HH. To this solution, 0.7 ml of absolute ethanol was added, and the cells were then stored at −20°C until use. Care was taken to avoid cell clumping by repeated and gentle pipetting of cells. After the fixation procedure, samples could be stored at −20°C for several months without affecting assay performance.

In situ hybridization. Typically, an aliquot containing the $3-5 \times 10^5$ fixed cells in a 1.5-ml microfuge tube was treated with 1 μl of 10% DEPC in ethanol per 50 μl of cell suspension for 15 min at room temperature to block the activity of endogenous RNase. The cells were then centrifuged at 300 *g* and incubated with 0.5% Tween 20 in HH buffer (HH-T) for 5 min at room temperature. To this solution, one volume of 20 × SSC and two volumes of formamide were added, and the cells were aliquoted at $3-5 \times 10^5$ cells/tube to the required number of tubes. The cells were spun as above and incubated with a biotinylated V_H gene probe at a final concentration of 10 ng/ml in 10 μl of hybridization buffer (5 × SSC, 50% formamide, 0.1% SDS, and 500 μg/ml *Escherichia coli* RNA). The hybridization buffer as well as the subsequent washing step buffer were warmed to 45°C before addition to the cells. The cells were incubated with the probe overnight at 45°C on a rocking platform (200 rpm). After the overnight incubation, excess probe was removed by addition of 100 μl of hybridization buffer (without the V_H gene probe) and incubated at 45°C for an additional 45 min. The cells were centrifuged, resuspended in 100 μl of 0.1 × SSC and 0.1% SDS, and incubated at 45°C for 30 min to remove mismatched hybrids. After centrifugation, the cells were incubated with 100 μl of 5 μg/ml streptavidin-phycoerythrin (PE) (Fisher Scientific) or anti-digoxigenin-fluorescein Fab fragments (Boehringer Mannheim Biochemicals) in HH-T buffer at 45°C for 30 min. Next, the cells were washed with 400 μl of HH-T buffer with 0.1 × SSC and 0.1% SDS at 45°C for 2 min to remove nonspecifically bound streptavidin-PE or anti-digoxigenin-fluorescein Fab fragments, centrifuged, and resuspended in 0.5 ml of HH-T buffer for analysis.

Flow cytometry. The fluorescence and the light scatter of the cells were measured with a FACScan® (Becton Dickinson, San Jose, CA) with a 0.5-W 488-nm argon laser light. Either single- or two-color flow cytometry was performed. Signals from 2,500–5,000 cells were collected in list mode. Histograms were constructed with the signals from single cells selected by gating the forward and perpendicular light scatter fluorescence parameters. The data were analyzed using FACScan® research software (Becton Dickinson).

Results

Determination of the V_H gene sequence. Total RNA from IM-9 cells and bone marrow (BM) mononuclear cells from five patients with MM was amplified with sense V_H family specific and antisense Cγ primers. A single band of ~ 450 bp in size was noted for each PCR reaction using only one of the V_H family primers (Tables I and II). The PCR product ranged in size from 414 to 486 bp and included the entire V_H gene (Table II), and was then sequenced and compared with published V_H genes (14, 15). The cloned myeloma V_H sequence was then used to make sense and antisense RNA probes for in situ hybridization. A pair of specific sense and antisense primers around the CDR2 region (Fig. 1) was then designed and used to amplify and subclone the myeloma Ig CDR2 regions of IM-9 and MM patient WAD#35. Shorter 89- and 87-bp RNA probes, respectively, were obtained after translation of these subclones for the further studies of in situ hybridization.

Identification of IM-9 cells with an antisense RNA probe. To define the optimal conditions for detection of V_H expression by means of FISH and flow cytometry (FISH-FC), the initial experiments were carried out on the IM-9 myeloma cell line. The cell line synthesizes relatively high levels of IgG (11) and provides a convenient source of cells expressing a singular V_H gene sequence.

Table II. V_H Family Repertoire of the MM Patients and the IM-9 Cell Line and Size of Their PCR Products

	Ig type	V_H family	Size of first PCR product	Size of second PCR product
WAD#1	IgG	V_H1	414 bp	
WAD#12	IgG	V_H3	440 bp	
WAD#35	IgG	V_H3	474 bp	87 bp
WAD#20	IgG	V_H4	425 bp	
WAD#25	IgG	V_H6	456 bp	
IM-9 cell line	IgG	V_H3	486 bp	89 bp

GenBank numbers are as follows: WAD#1, x79158; WAD#12, x79169; WAD#20, x79177; and WAD#25, x79182. WAD#35 and IM-9 cell line have been submitted to GenBank.

The fluorescent histogram profile of the IM-9 cell line hybridized with the biotinylated RNA probes is shown in Fig. 2. When the specific antisense RNA probe was used for hybridization, a very strong signal was obtained which averaged approximately 124-fold higher than the sense probe control value. Increased signal above background staining was detected on all of the IM-9 cells using the antisense probe.

To prove that intact RNA probes are required for generating the higher signal, the IM-9 sense and antisense probes were treated with RNase, and the products were hybridized with the IM-9 cells (Fig. 3). The RNase abolished most of the antisense signal, indicating that intact antisense RNA probe is required for hybridization.

Identification of myeloma cells with antisense RNA probes. To examine if this technique could be applied to clinical specimens, we constructed RNA probes from the V_H gene sequence expressed in five MM patients. The MM patients' BM mononuclear cells were hybridized to their respective sense and antisense RNA probes, and cell labeling with the antisense probe was markedly increased in each case from 9- to 28-fold compared with the no probe control sample. Fig. 4 represents our results for patient WAD#20. This patient had 90% plasma cells on the lymphocyte/plasma cell gated sample that was assayed, and this explains why almost all of the cells were labeled with the antisense probe. These results from the cell line and MM patient BM cells demonstrated the feasibility of using the FISH-FC technique for detection of V_H expression in individual cells.

Probe specificity assays. First, we wanted to determine if the RNA probes were at least V_H gene family specific. Consequently, WAD#1 bone marrow cells were incubated with its own V_H1 antisense probe, and myeloma cell labeling was compared with a control experiment using patient WAD#12's V_H3 antisense probe (irrelevant antisense control). The patient WAD#1's own antisense probe stained with 22-fold higher fluorescent intensity, indicating that the detection of V_H gene expression is V_H family specific (Fig. 5). Some overlap of fluorescence staining intensity existed, but this may be explained by the presence of 20% normal cells in WAD#1's bone marrow sample.

Since most of the V_H germline gene sequence is homologous

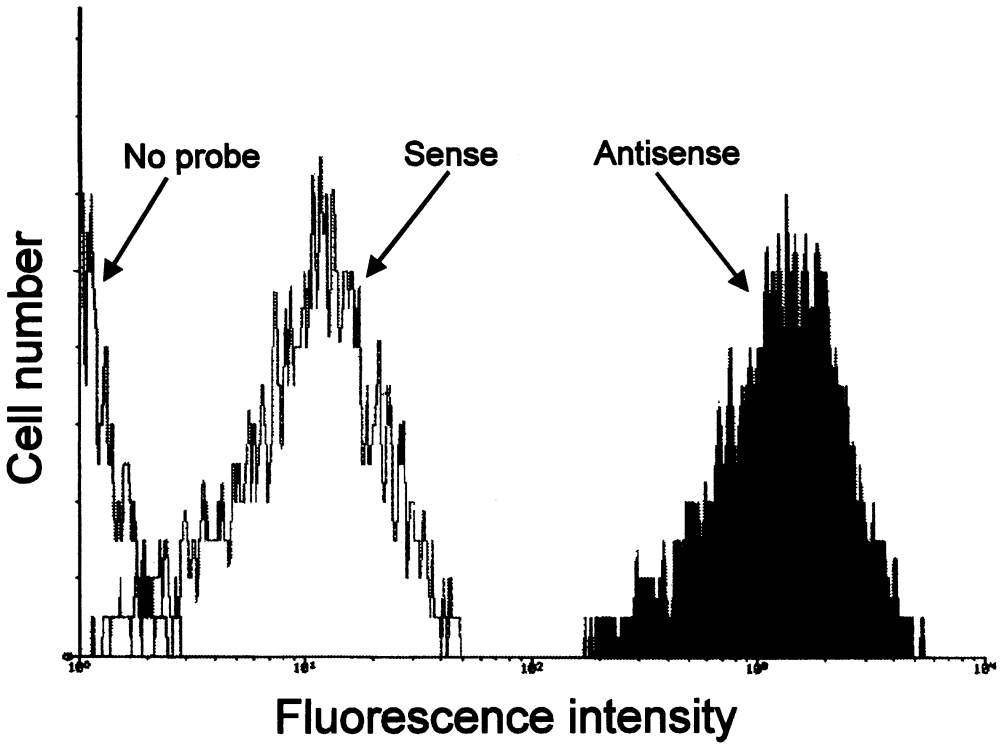

Figure 2. V_H expression by the IM-9 cell line as detected by RNA probe hybridization. IM-9 cells were hybridized with biotinylated sense or antisense RNA probes or a no probe control. The RNA probes were produced by transcription of the V_H gene sequence after insertion into a PCR script vector as described in Methods. After a washing step, streptavidin-PE was added and 2,500–5,000 cells were analyzed by a FACScan® in list mode after gating to exclude cell clumps and debris.

even among V_H gene family members, we believed that smaller RNA probes would be more specific for the myeloma gene sequence. Therefore, shorter RNA probes encompassing the CDR2 region were made for the IM-9 cell line and for patient WAD#35 for further study. The CDR2 region was chosen because this area is the most heterogeneous among V_H gene members. When results from cell labeling using the different length probes were compared, the mean fluorescent intensity given by the shorter antisense RNA probe was much higher than with the longer antisense probe after comparison with the no probe control background value (Fig. 6). This improvement may also be due to increased efficiency of RNA probe cell entry because of the smaller size of the shorter RNA probe.

Both the IM-9 cell line and the myeloma cells from patient WAD#35 express Ig derived from the same V_H gene family (V_H3). Consequently, their V_H gene sequences are quite similar although there is only 80% homology within the region encompassed by the CDR2 probes (Fig. 1). To assay for RNA probe gene specificity, both CDR2 antisense probes derived from the IM-9 and WAD#35 myeloma cells, respectively, were hybridized with the IM-9 cells. It was found that the irrelevant WAD#35 antisense RNA probe showed an approximate 19-fold lower signal than the matching IM-9 antisense RNA probe (Fig. 7). This illustrates that the detection of V_H gene expression is specific even for different members of the same V_H family.

Probe sensitivity. To test the ability of FISH-FC to quantitate the percentage of cells that express a particular V_H gene in a mixed heterogeneous cell population, known numbers of IM-9 cells were mixed with normal donor's BM mononuclear cells by serial dilution. Eight samples containing IM-9 cells at various percentages (100, 75, 50, 25, 10, 5, 2, and 1%) were analyzed using the IM-9 V_H CDR2 antisense probe in addition to a control sample containing only normal BM cells. The pure cell populations were used to define the cursor position that best differentiated between a positive and a negative cell, and this was set at the location where 99% of IM-9 cells would have higher fluorescence intensity staining than the normal BM cells. When these samples were analyzed, the calculated percent contamination of the eight samples by FISH-FC was 99, 73.6, 48.6, 24.3, 10.2, 4.9, 2.0, and 1.1% (Fig. 8). In every case, there was an easily identified population of cells with significantly higher fluorescence staining presumably representing the cells from the IM-9 cell line. Therefore, this FISH-FC technique can effectively identify the target cells which express a particular V_H gene in mixed populations with a high degree of accuracy.

Discussion

It is important to be able to specifically identify and separate malignant cells in mixed populations in human lymphoid malignancies for basic research and clinical applications. Previously developed approaches of studying gene expression will not permit further purification of the malignant cells due to (*a*) cell disruption (Northern blot and colony blot assays), or (*b*) cell fixation to slides (FISH) (16–18). By combining the specificity of fluorescent in situ hybridization with the advantages of flow cytometry, the FISH-FC technique described here can detect specific V_H gene expression while cells remain in suspension. This allows further studies on the cells of interest to be performed including additional FACS® analysis using different monoclonal antibodies and also cell purification by FACS® sorting.

In this report, the IM-9 cell line and five MM patients' bone marrow cells were hybridized with their respective biotinylated antisense RNA probes and demonstrated positive staining by FACS® analysis. Control assays using either a sense probe or no probe before streptavidin-PE exposure gave only background signals. We have also demonstrated the specificity of this FISH-

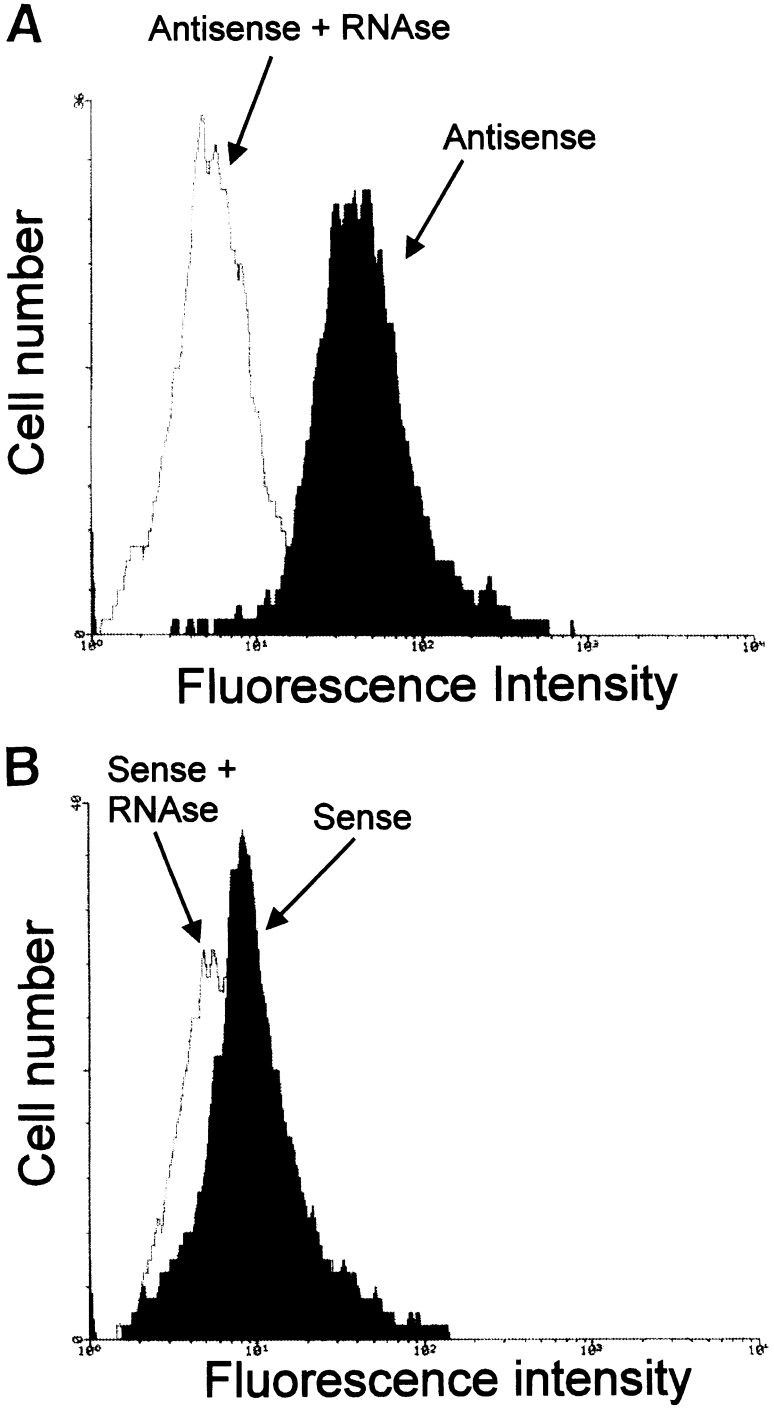

Figure 3. IM-9 sense and antisense probes were compared with RNase-treated probes for their ability to detect IM-9 cell V_H gene expression. Cells were analyzed as described in Fig. 2. The RNase treatment reduced the labeling of the antisense probe to background levels (*A*) comparable with sense and RNase-treated sense probe controls (*B*).

FC technique in detecting either the V_H gene family or the more specific V_H gene expressed by a B cell using V_H gene specific antisense probes. Ravichandran et al. (5) have used a FISH technique with longer murine V_H RNA probes from previously synthesized plasmids to demonstrate expression of V_H family genes in murine cell lines and normal murine spleen cells. In our study, we demonstrate the ability to generate patient specific V_H antisense RNA probes of shorter length (80–90 bp) which can effectively identify malignant cells in a human B cell malignancy, such as MM, with a higher degree of specificity.

The V_H gene segment includes the first and second complementarity determining regions and three framework regions. The CDRs encode for the regions of the Ig protein which confer antigen binding specificity and display considerable hypervariability compared with the surrounding framework regions which are usually more conserved within each V_H family (19). Therefore, sequences containing the CDR2 were used as templates to synthesize biotinylated RNA transcripts to improve specificity of the antisense probes. We have sequenced the Ig gene produced by myeloma cells from 48 patients and found that the CDRs contain the highest degree of somatic mutation (averaging 14.1%) after comparison with germline V_H genes (8). Consequently, RNA probes derived from here should maximally reduce the homology with other irrelevant V_H sequences. The

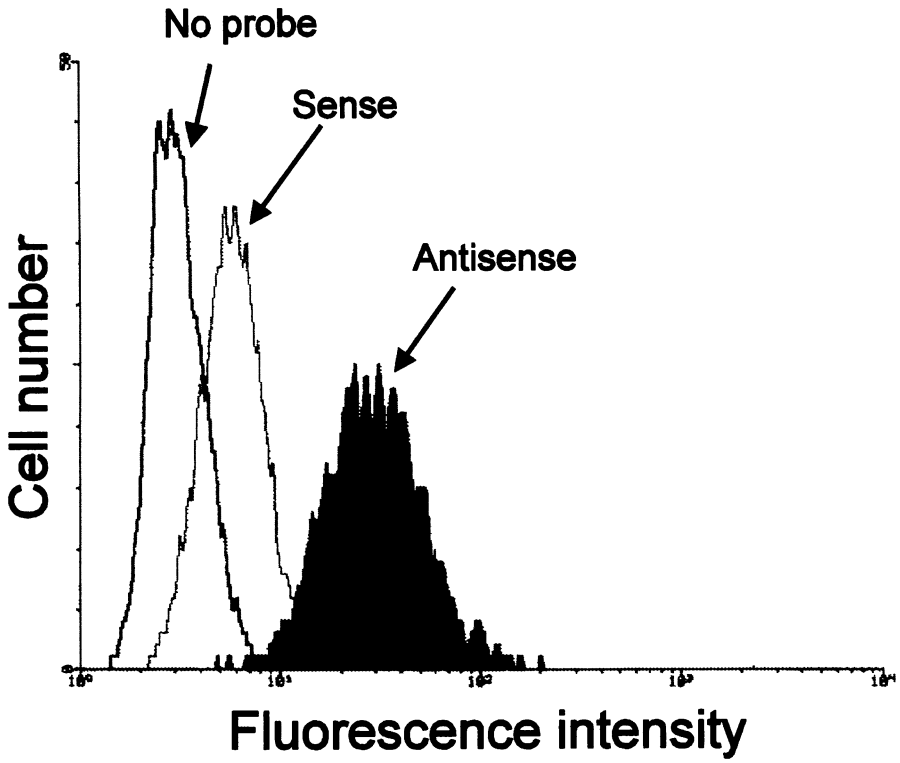

Figure 4. Detection of V_H expression in the BM cells from a MM patient. MM patient WAD#20's sense and antisense RNA probes or a no probe control, respectively, were hybridized with the patient's BM mononuclear cells. As this patient had a high tumor burden, most of the lymphocyte gated cells stained above background with the antisense probe.

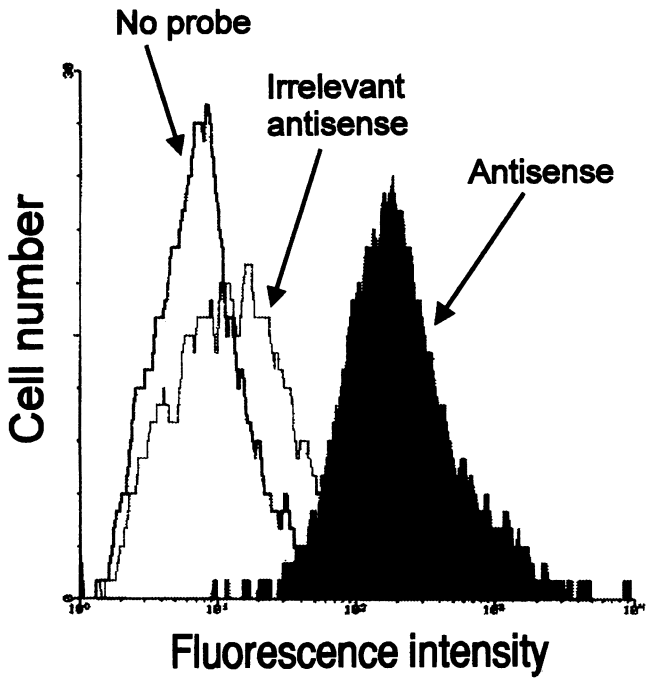

Figure 5. Specificity of V_H gene detection using WAD#1 and WAD#12 antisense RNA probes which were derived from different V_H gene families, V_H1 and V_H3. Bone marrow cells from WAD#1 were hybridized with either the WAD#1 antisense probe, WAD#12's antisense probe (irrelevant antisense), or a no probe negative control. The mean fluorescent intensity obtained using the WAD#1 antisense probe is 22-fold higher than that obtained with the irrelevant antisense control.

lengths of the CDR2-derived templates are ~ 80–90 bp, which has been shown to be the optimal length for RNA probes used for fluorescent in situ hybridization (4). It is possible that probes to the CDR3 of the heavy chain sequence would be even more specific since this is the region of greatest Ig sequence diversity. However, we did not choose the CDR3 region as a probe in our study primarily because its length is too short for optimal PCR and RNA transcription. This inherent problem was demonstrated by the fact that the fluorescent signal with the CDR3 probe was markedly weaker than that of the CDR2 probe when compared in our lab using the IM-9 cell line (data not shown). To overcome the difficulty in using the shorter CDR3 probe, it may be possible to extend the length of the probe into the framework regions. However, because of the more conserved nucleotide sequences, this may compromise probe specificity. In addition, we have found a lack of N region insertions in the V_H-D and D-J_H junctions and the CDR3 length to be short (average length 30 bp) in myeloma (20). As a result, this region is less optimal than CDR2 in developing antisense probes in myeloma.

The abolition of hybridization signal by RNase treatment suggests the formation of RNA/RNA hybrids between the antisense biotinylated RNA probe and the mRNA within these myeloma cells. This observation is similar to other reports using non-Ig antisense RNA probes (4). These prior studies and our observations suggest that the detection of fluorescent signal is dependent upon the formation of a complex involving both the target mRNA and the antisense probe.

Although we do not have direct evidence as to whether clonal cells with suboptimal Ig production would remain detectable with FISH-FC, in theory only a few Ig mRNA molecules should be detectable by this assay assuming 100% hybridization efficiency (21). When the IM-9 cell line was used, the majority of these cells expressed fluorescence intensity above the highest

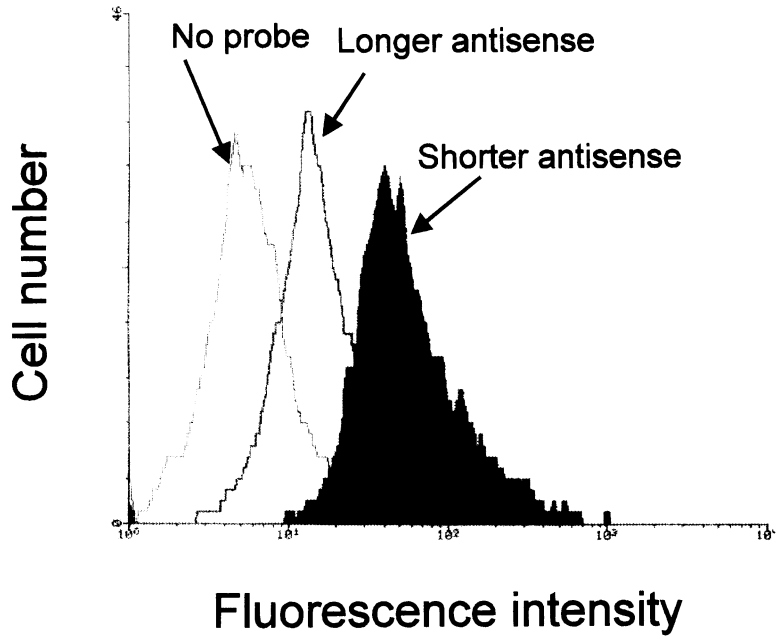

Figure 6. Comparison of signal intensity using shorter and longer RNA antisense probes. Staining of IM-9 cells with the longer 486-bp RNA probe was compared with staining using a shorter 89-bp RNA probe which is complementary to the CDR2 region. The fluorescence intensity given by the shorter antisense RNA probe is threefold higher than the longer antisense RNA probe compared with the no probe.

staining normal BM cells, although there remains a 1% overlap possibly due to IM-9 cells with reduced Ig gene production. This phenomenon has been described in many myeloma cell lines and may also occur in vivo, which could limit the ability to identify every malignant cell. Despite this limitation, IM-9 cell contamination was accurately quantitated by FISH-FC in multiple samples, and this technique should be especially valuable in the identification and selection of unequivocally malignant cells in a mixed cell population.

Previous studies in myeloma have used the identification of cells expressing the same light chain as that expressed by the paraprotein as a way to identify malignant cells in myeloma patients' bone marrow and peripheral blood (6, 22). Using routine in situ hybridization with biotinylated probes to light chain mRNA, Akhtar and co-workers (6) have identified clonal cells in myeloma bone marrow tissue sections. This is unquestionably less specific since up to one half of the normal B cells could be expected to use the same light chain as the myeloma cells. By using V_H CDR2 specific probes, the FISH-FC technique should permit a more precise identification of the malignant cells in these patients. Moreover, this approach may allow the detection of clonal cells at earlier stages of B lymphoid differentiation since preclass switch cells, which typically rearrange and express their V_H gene sequence before light chain rearrangement, could be identified with this new technique.

We are currently using this technique to simultaneously

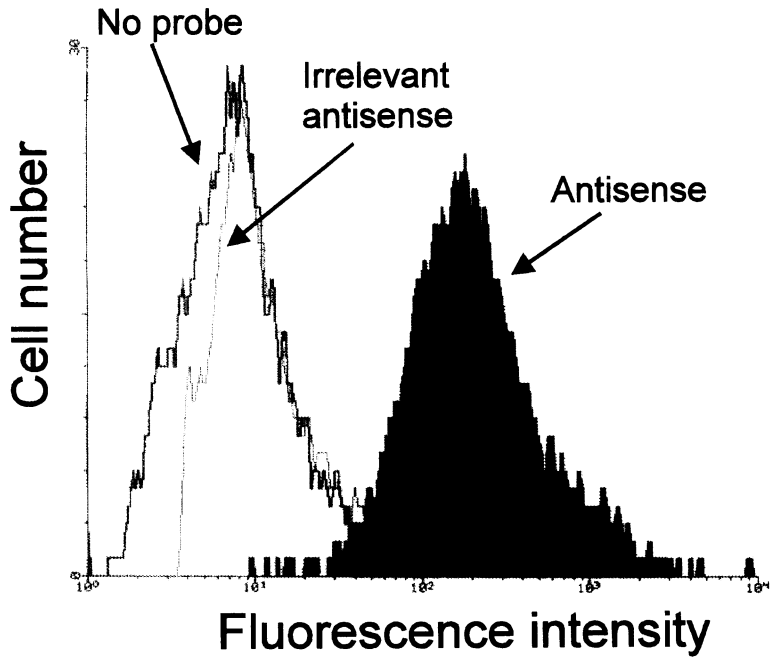

Figure 7. Specificity of V_H gene detection using CDR2 RNA antisense probes derived from members of the same V_H gene family, V_H3. IM-9 cells were incubated with either the IM-9 CDR2 antisense probe, WAD#35 CDR2 (irrelevant) antisense probe, or a no probe control. The mean fluorescent intensity using the IM-9 cell line antisense probe is 19-fold higher than that obtained using the irrelevant V_H CDR2 antisense probe.

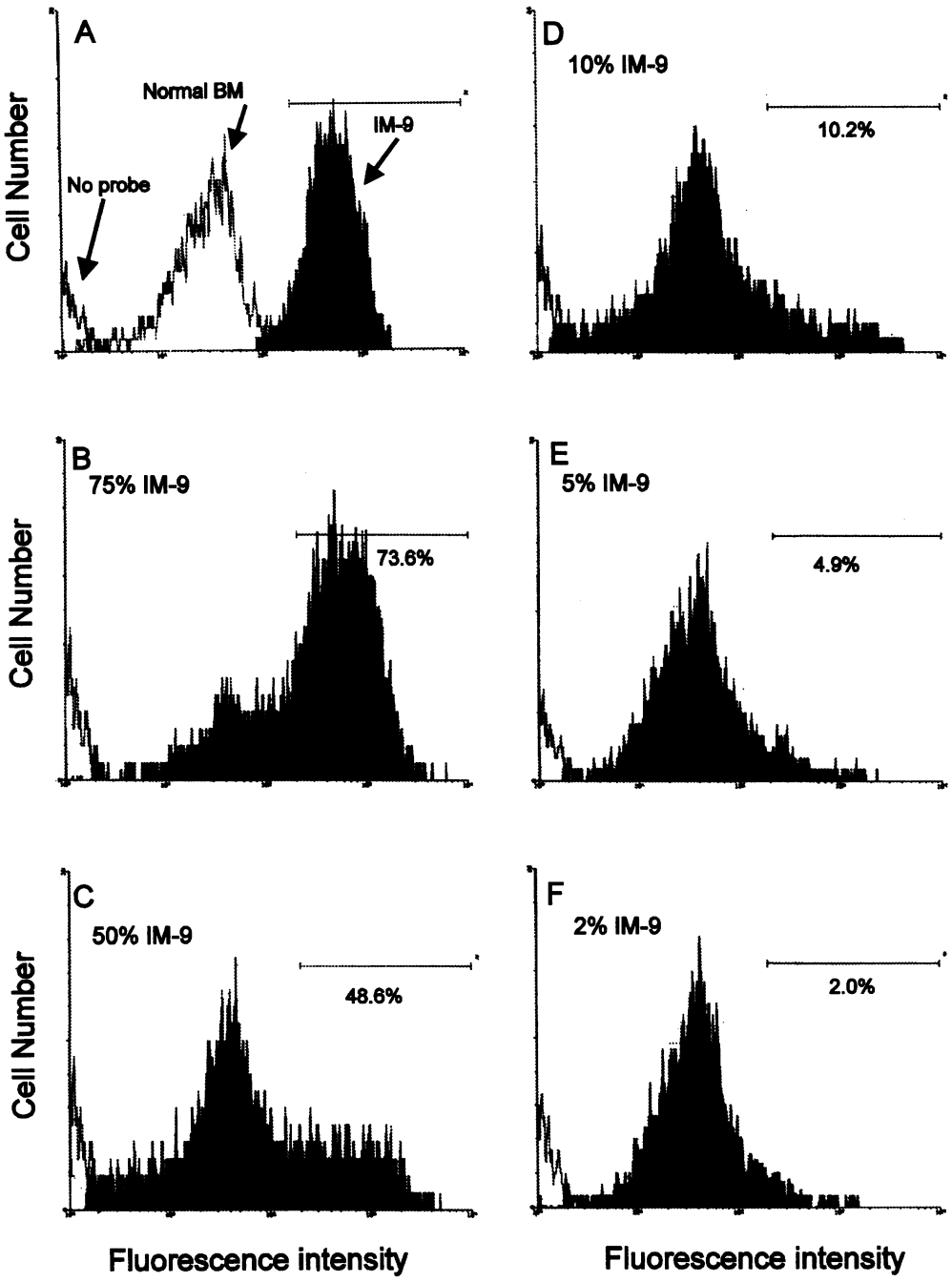

Figure 8. Detection of V_H mRNA expression in mixed cell populations. IM-9 cells were serially diluted with normal BM cells to various dilutional concentrations and, respectively, hybridized with the IM-9 V_H CDR2 antisense probe. *A* depicts the fluorescent histograms obtained using pure IM-9 cells or pure normal BM cells with the antisense probe in addition to a control using IM-9 cells without a probe (*No probe*). A minimum fluorescent intensity which included 99% of the IM-9 cells was used to define positive cell staining. *B–F* show the percentage of cells that express the IM-9 V_H CDR2 gene in the different cell mixtures. The percentage of positive cells as recognized by antisense reactivity is shown in each panel.

detect expression of a number of cell surface antigens on the malignant cell's surface in myeloma patients. Studies identifying the phenotype of all members of the malignant clone in myeloma and other B cell malignancies are of increasing importance as more attempts are made to eliminate these cells by both ex vivo purging for use in autologous transplantation (23) and in vivo immunotherapeutics (24). For example, previous studies have suggested that the cell surface marker CD38 is expressed at very high levels on malignant plasma cells in myeloma patients (25). With the FISH-FC technique, we are determining whether all of the malignant cells in these patients actually express high levels of this marker using a FITC-conjugated anti-CD38 antibody in combination with the biotinylated antisense V_H probe and streptavidin-PE by means of two-color flow cytometry (our unpublished data). We are also using a similar approach to detect whether the CD34 antigen, a marker of early hematopoietic precursors, is expressed on any malignant cells in myeloma patients. It may also be feasible to study multiple antigens simultaneously on a specific V_H gene–expressing B cell subpopulation. This may be accomplished by adding an antibody conjugated to a marker with a different fluorescence pattern than PE or FITC such as allophycocyanin (26).

It is also possible that this technology can be used to determine the expression of other mRNA transcripts in the malignant B cell subpopulation when it is mixed with nonmalignant cells. For example, detection of cytokine gene and oncogene expression may be specifically identified in the malignant cells with the

FISH-FC technique. Detection of IL-1 expression in different murine tissue samples and human monocytes has been accomplished with hybridization using ^{32}P- or ^{35}S-labeled cDNA probes (27, 28). Past attempts to quantitate oncogene in situ expression specifically in myeloma cells have involved determination of c-*myc* expression by in situ hybridization procedures with a ^{35}S-labeled c-*myc* antisense probe on morphologically identified myeloma plasma cells (29). These previous attempts to use in situ hybridization procedures in cytokine and/or oncogene expression studies have been limited by the inability to precisely identify the malignant cells and by the requirement of cell fixation on the slides.

In summary, the FISH and FC method should allow the precise identification and separation of clonal B cells in a mixed cell population. This technical advance will be important in further characterizing the specific phenotype and genotype of cells in both malignant and nonmalignant clonal B cell disorders. Moreover, this technique should also be applicable to nonlymphoid cells in which the identity of a specific cell population can be ascertained by the expression of a unique gene.

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