Methods to Detect P-Glycoprotein-associated Multidrug Resistance in Patients’ Tumors: Consensus Recommendations


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ABSTRACT

Multidrug resistance (MDR), especially that associated with overexpression of MDR1 and its product, P-glycoprotein (Pgp), is thought to play a role in the outcome of therapy for some human tumors; however, a consensus conclusion has been difficult to reach, owing to the variable results published by different laboratories. Many factors appear to influence the detection of Pgp in clinical specimens, including its low and heterogeneous expression; conflicting definitions of detection end points; differences in methods of sample preparation, fixation, and analysis; use of immunoreagents with variable Pgp specificity and avidity and with different recognition epitopes; use of secondary reagents and chromogens; and differences in clinical end points. Also, mechanisms other than Pgp overexpression may contribute to clinical MDR. The combined effect of these factors is clearly important, especially among tumors with low expression of Pgp. Thus, a workshop was organized in Memphis, Tennessee, to promote the standardization of approaches to MDR1 and Pgp detection in clinical specimens. The 15 North American and European institutions that agreed to participate conducted three preworkshop trials with well-characterized MDR myeloma and carcinoma cell lines that expressed increasing amounts of Pgp. The intent was to establish standard materials and methods for a fourth trial, assays of Pgp and MDR1 in clinical specimens. The general conclusions emerging from these efforts led to a number of recommendations for future studies: (a) although detection of Pgp and MDR1 is at present likely to be more reliable in leukemias and lymphomas than in solid tumors, accurate measurement of low levels of Pgp expression under most conditions remains an elusive goal; (b) tissue-specific controls, antibody controls, and standardized MDR cell lines are essential for calibrating any detection method and for subsequent analyses of clinical samples; (c) use of two or more vendor-standardized anti-Pgp antibody reagents that recognize different epitopes improves the reliability of immunological detection of Pgp; (d) sample fixation and antigen preservation must be carefully controlled; (e) multiparameter analysis is useful in clinical assays of MDR1/Pgp expression;

(f) immunostaining data are best reported as staining intensity and the percentage of positive cells; and (g) arbitrary minimal cutoff points for analysis compromise the reliability of conclusions. The recommendations made by workshop participants should enhance the quality of research on the role of Pgp in clinical MDR development and provide a paradigm for investigations of other drug resistance-associated proteins.

INTRODUCTION

Considerable effort has been expended to design and execute clinical trials testing methods for the detection and reversal of MDR—i.e., form of resistance to “natural product” anticancer agents—in cancer patients (1–9). Because these studies have not yielded consistent results in terms of Pgp expression and reversal of MDR (8–14), and given the continued interest in such research, it seems appropriate to ask whether the problems encountered might reflect inadequate experimental support. In general, the MDR1 gene and its product, Pgp, are overexpressed de novo in certain malignancies (1, 2, 15) and after chemotherapy with Pgp substrates in others (16–19). However, aberrant expression of the protein does not always correlate with the clinical status of the patient or the outcome of therapy (e.g., Refs. 15 and 20), nor have attempts to circumvent clinical MDR with Pgp “modulators” produced uniform results (8–14). Because of these inconsistencies, which no doubt stem from difficulties in detecting Pgp, as well as other biochemical and pharmacological factors contributing to resistance, the role of this protein in clinical MDR remains unclear.

A major problem in assessing the significance of MDR1 and Pgp overexpression in clinical MDR has been the variability in measurements of these factors (10). Although most laboratories can reliably detect high levels of expression of both MDR1 and Pgp, regardless of the method or reagent(s) used, lower levels of expression have proved more difficult to quantify. Variable quantitative results have been reported for similar clinical specimens by different laboratories using immunostaining; the use of flow cytometry, Northern blotting, RNase protection, RNA in situ hybridization, Western blotting, and r-PCR (1–6), and few studies have attempted to compare multiple methods (e.g., Refs. 3 and 4). Even with the same assay (e.g., immunostaining) and disease, findings have varied according to the anti-Pgp antibodies and secondary detection systems selected (16, 20). Moreover, the cross-

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3 Dr. Parham is now at the University of Arizona College of Medicine, Tucson, AR.

4 Dr. Kuttesch is now at the M. D. Anderson Cancer Center, Houston, TX.

5 The abbreviations used are: MDR, multidrug resistance; AML, acute myeloid leukemia; KCC/CHC, immunohistochemistry/immunochemistry; Pgp, P-glycoprotein; PE, phycoerythrin; S.N., signal/noise; r-PCR, reverse transcription-PCR; NCI, National Cancer Institute; ALSAC, American Lebanese Syrian Associated Charities; SWOG, Southwest Oncology Group.
The detection of MDR1 and Pgp in clinical specimens has relied heavily on antibody and cDNA probes (Refs. 1–6 and references therein; Ref. 24). Although immunostaining is routinely applied in pathology laboratories, its use to detect Pgp in tumor specimens poses special problems. Rather than asking whether a specimen contains or lacks Pgp, as is common when attempting to find a diagnostic marker, one must quantify the extent of Pgp expression, which in experimental systems is directly correlated with the degree of tumor cell MDR (25–27), although quantifying the numbers of Pgp molecules required to confer drug resistance to a tumor cell remains elusive. Thus, the theme of the workshop became “improved detection of MDR1/Pgp in tumor specimens through comparison of standard techniques.” Specific objectives were to (a) test the logistics of tumor and sample distribution, Pgp detection methods, and data interpretation; (b) identify pitfalls in the system; (c) determine if a resource laboratory can distribute samples with adequate preservation of Pgp; and (d) determine if immunostaining and other methods and the interpretation of results show substantial variability among and within laboratories. The overall goal was to lay the groundwork for moving MDR1/Pgp from the laboratory to the clinic, ultimately as a target for specific therapies. The results and recommendations presented here summarize the entire agenda of the St. Jude MDR Workshop on Methods to Detect P-Glycoprotein-associated Multidrug Resistance.

MATERIALS AND METHODS

Cell Lines. Well-characterized cell lines served as calibration kits in each of the comparative trials of the workshop. As models for hematological cancers, we used the 8226 myeloma cell line and its well-characterized MDR-expressing derivatives 8226/Dox 1, 6, and 40 (27), which were selected for increasing resistance to doxorubicin (Dox 1 and 6 express low levels of resistance). The solid tumor model was the KB cell line and sublines selected for increasing levels of MDR expression [KB3-1, KB8-5-11 (26), and KBV1 (28)]. Each cell line was provided to participating investigators in all trials. Ultimately, the lines served as calibration standards for the trials involving clinical samples. Depending on the objective of the trial, cells were provided either as fixed material (cytospin preparations, 8226 cells; paraffin sections, KB cells) or as live cells in culture (8226 cells).

Antibodies. The seven anti-Pgp antibodies used in comparative analyses (Table 1) were generous gifts of commercial vendors: C219, C494, ISB-1, and 4E3 through Dr. Ronald J. Casiati (Signet Laboratories, Inc., Dedham, MA); UIC-2 through Drs. Eugene Mechetner and Gregory Reyes (Ingenix, South San Francisco, CA); MRK-16 through Colin Getty (Kamiya Biochemicals, Thousand Oaks, CA); and HYB-241 through Dr. Lana Grauer (Hybritech, La Jolla, CA). Fluorescein- or biotin-labeled second antibodies and enzyme-chromogen detection systems were obtained from standard commercial sources, and each participating laboratory used its own “second-step” reagents and detection methods. The characteristics of these antibodies, their original citations, the concentrations provided by the vendors, and the concentrations used in the trials are given in Table 1.

Antibodies were titrated by three of the workshop participants; they were titrated for cytospin and histological preparations by Dr. T. M. Grogan (Tucson, AZ), using 8226 cells; for histological sections by Dr. C. Cordon-Cardo (New York, NY), using normal kidney and renal tumors; and for flow cytometry by Dr. C. L. Willman (Albuquerque, NM), using transformed myeloid blast cells. Once approximate titrations were known, the commercial vendors provided the same lot of each antibody for all investigators, with recommendations for dilutions. Antibody optima for immunohistochemical assays were determined by standard microscopic morphometric grading, and optimal S:N ratios were assessed by image analysis (35).

Solid Tumor “Buttons.” Vinblastine-resistant and vinblastine-sensitive KB cells were prepared by Dr. T. M. Grogan as formalin-fixed, paraffin-embedded solid tumor specimens to test histological methods in Trial 2. Further, in Trial 4, “sausages” of KB3-1, KB-8-5-11, and KBV1 cells were placed on the same slides as the clinical specimens to view positive and negative controls simultaneously.

Immunocytochemical and Immunohistochemical Staining. Workshop participants were free to use their standard methods for these procedures, all of which have been published elsewhere (summarized in Table 2).

Table 1 Characteristics of workshop antibodies

<table>
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<tr>
<th>Characteristic</th>
<th>4E3</th>
<th>C494</th>
<th>C219</th>
<th>JSB-1</th>
<th>UIC2</th>
<th>MRK16</th>
<th>HYB241</th>
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<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
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a Spaces indicate missing information or no further manipulations.

b AP, affinity purified.

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Table 2. Summary of methods used by workshop participants

<table>
<thead>
<tr>
<th>Method</th>
<th>Investigators</th>
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<td>Twentyman</td>
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<td>IHC</td>
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<td>Frozen sections</td>
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<td>Paraffin blocks</td>
<td>Parham, Kuttesch</td>
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<td>Antigen retrieval</td>
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<td>Flow cytometry</td>
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<td>Pgp detection</td>
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<td>Pgp function (dye or drug efflux)</td>
<td>Bates, Fojo</td>
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<td>r-PCR</td>
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<td>49</td>
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<td></td>
<td>Willman</td>
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<td>Multiple methods</td>
<td>Wanske, Sikic</td>
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<td></td>
<td>Dietel</td>
<td>51-54</td>
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<td></td>
<td>Bates, Fojo</td>
<td>3</td>
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participants by overnight courier (North America) or 2-day delivery (Europe). The expression of Pgp was characterized by the reference laboratory as high, meaning positive staining for MRK16 (MRK16+) and the ability to efflux either DIOC2 or rhodamine 123 (efflux +), as detailed in Ref. 16, at levels similar to those seen with 8226/Dox6 cells (efflux +, >95% M1 blasts (~Dox6)); intermediate, meaning MRK16+/efflux +, >90% blasts (<Dox6); or low, meaning gated blasts MRK16−/efflux −, <90% blasts (<80%) with contaminating T cells (samples collected from peripheral blood).

Samples of renal cell carcinoma and normal kidney tissue from the same case were either embedded in OCT compound (Miles, Inc., Elkhart, IN) and frozen in liquid nitrogen or fixed in buffered formalin and embedded in paraffin. Tissue sections for immunohistochemical studies were distributed to participating institutions by overnight (North America) or 2-day (Europe) delivery.

For immunohistochemical testing of rhabdomyosarcoma, a single case that had shown only focal Pgp staining was selected. Paraffin sections of formalin-fixed tissue and cryostat sections of fresh tissue preserved at −70°C were prepared, with care taken to avoid thawing of the frozen sections. The specimens were then shipped to the participants on dry ice by overnight delivery.

Data Collection and Analysis. The Cellular Analysis System 200 and the Cell Measurement 2.0 program (Becton Dickinson, Mountain View, CA) were used in quantitative imaging (35). The 2.0 program measures several cellular properties, including nuclear features (nuclear size and shape, and summed and average absorbance) and cytoplasmic features (size, shape, and summed absorbance). Because of the nonnuclear location of Pgp, we used the summed cytoplasmic density measurement, centering the cells of interest in the microscopic field and analyzing 100 consecutive cells. The S:N ratio was calculated as the mean of the summed cytoplasmic densities of 100 KBV-1 cells divided by the mean of the summed cytoplasmic densities of 100 KB3–1 cells.

References for flow cytometric methods for measuring Pgp levels and functional activity are cited in Table 2, as are references for r-PCR, primers for MDRI, and control probes.

RESULTS AND DISCUSSION

Trial 1. Logistics of Sample Distribution and Pgp Detection by Immunocytochemistry. For this trial, we used 8226 cell lines as models of hematological tumors for standardization and comparison of Pgp detection methods. Coded cytospin preparations on glass slides, fixed with acetone (4°C), were sent from Tucson, AZ, to participating laboratories by overnight express, to be stained and analyzed within 24 h, with repeat analyses performed in each of the next 3 days. Stained slides were initially analyzed microscopically by individual laboratories, and then sent back to Tucson for quantitation by image analysis. A representative slide is shown in Fig. 1. The apparent cytoplasmic staining of Pgp in these cells is an artifact of the cytochemistry method: total cells, not sections, are examined from the top, so it appears to be cytoplasmic staining, when in fact it is plasma membrane staining. Virtually all of the 11 institutions participating in this trial were able to demonstrate appropriate Pgp staining density in the cell lines, although there was considerable variation among institutions, especially when the parental drug-sensitive cell line and the
two cell lines with low levels of resistance, Dox1 and Dox6, were used (Fig. 2). Thus, the data underscore the importance of true negative controls. Indeed, when results were quantified as S:N ratio, the value for Dox40 was consistently higher than that for Dox1 (Fig. 3). Staining of consecutive slides over 3 days yielded highly reproducible staining densities (data not shown), indicating that under these conditions of fixation and transportation, Pgp "travels well."

In this trial, 8226 cells in acetone-dipped cytospins provided a reliable transferable standard (reproducible results obtained for at least 3 days) and therefore should be useful in multi-institutional studies of cellular Pgp. These fixed cells can be sent thousands of miles to many different laboratories without appreciable effects on the results of qualitative and quantitative measurements of Pgp.

At the outset of the workshop, we questioned whether relative density determinations based on S:N ratios would provide reliable measurements for assay optimization among large numbers of institutions. Our findings suggest that by setting the S:N ratio as shown in Fig. 3, A and B, one can establish a quantitative benchmark that will facilitate comparisons among institutions. This is a novel observation that will be the subject of a separate publication.

**Trial 2. Evaluation of KB Cells as Models of Solid Tumors in Standard Immunohistochemical Assays of Pgp Expression.** We deemed it important to have a solid tumor cell line resource that would allow testing of the effects of fixation, antigen retrieval, and, ultimately, optimization of histological Pgp assays. To adapt Pgp+ and Pgp− KB cells for this purpose, we subjected them to the entire fixation procedure. To address the issue of poor tissue fixation, a common problem in clinical evaluations of MDR, we prepared a tissue-fixation sausage consisting of pellets of variably fixed KB3−1 (Pgp−) and KBV1 (Pgp+) cells that were individually embedded in master paraffin blocks and sectioned as shown in Fig. 4. The purpose was to develop internal Pgp+ and Pgp− validation controls for clinical specimens with unknown Pgp expression. Thus, KB3−1 (Pgp−) and KBV1 (Pgp+) cells were grown in cell culture, centrifuged, fixed in colloidion bags and embedded with paraffin for histological sectioning, after which samples were coded and provided to all participating laboratories for Pgp staining and analysis. Investigators were asked to use an antibody (e.g., JSBI or C494) that is known to stain Pgp in formalin-fixed, paraffin-embedded tissues (24, 31, 37), although the recognition of other epitopes by C494 is acknowledged (22).

From the immunohistochemical staining results in Fig. 5A, it is clear that Pgp can be detected by this method in KB cells. Image analysis of cells stained with C494 antibody in two different laboratories (laboratories 1 and 10) yielded sizable S:N ratios for KBV1 (Pgp+) and KB3−1 (Pgp−) cells. In contrast, a third laboratory (laboratory 5) used a second antibody, HYB241, not known to react with Pgp under these conditions of fixation, that produced a weak signal (Fig. 5B).

Thus, although we did not solve all of the problems inherent in antibody staining for Pgp in solid tumors, trial 2 accomplished several important goals. First, it validated KB cells as a legitimate solid tumor model that can be used productively without the expense and technical difficulties of xenograft models. Second, and perhaps most important, we were able to show that a Pgp± control sausage placed on the same slide with a clinical specimen of unknown Pgp content will permit direct validation of Pgp

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6 L. Rimsza and T. M. Grogan, manuscript in preparation.
staining. The success of this method in trial 2 led to its adoption in trial 4, described below.

**Trial 3. Comparative Test of Different Methods for Measurement of Pgp and MDR1.** A major aim of the workshop was to compare results of immunostaining and flow cytometric identification of Pgp, flow cytometric assessment of Pgp function by dye efflux, and PCR measurement of MDR1 mRNA expression. This goal was pursued in trial 3 with use of suspension cultures of 8226/S and 8226/Dox cell lines individually and as admixtures. Briefly, coded flasks of 8226/S and 8226/Dox lines were provided to investigators for tests of specificity. Sensitivity testing of assays was conducted with admixtures of 90% S and 10% Dox40 cells and 99% S and 1% Dox40 cells. Investigators were encouraged to apply whatever methodology they preferred to determine the amount or level of Pgp or MDR1 expression, as well as Pgp function. The value of standardized antibody usage was assessed by testing both a single antibody across multiple methods and multiple antibodies within a single method (data not shown).

Results of these multiple comparisons are summarized in Fig. 6. The larger number of tests performed with immunostaining compared to flow cytometry, rt-PCR, or dye efflux can be attributed to the use of as many as seven different anti-Pgp antibodies by different laboratories. There was no evidence in any of the subtrials that a particular antibody provided a detection advantage in immunostaining procedures. It is clear that each assay was capable of correctly identifying drug-sensitive cells, as well as those with strong expression of MDR. Perhaps of greater importance, the assays were sufficiently sensitive to distinguish marginal situations in which drug-resistant cells made up only 1% or 10% of the total cell population (Fig. 6, E and F); however, the variable detection rates with immunostaining, flow cytometry, and dye efflux suggest that these methods may yield a significant percentage of false negative results. The same caveat applies to immunostaining and flow cytometry used with a cell line expressing low levels of Pgp, and to all assays used with a cell line characterized by faint expression of Pgp and the MDR1 gene. Some laboratories relied on PCR to perform a quantitative analysis. Indeed, results from one participating laboratory demonstrated that PCR could quantitate a >160,000-fold range of expression of MDR1 mRNA, ranging from 0.01 in 8226/S to 1692 in 8226/Dox40. However, the sensitivity of the PCR assay in most situations is offset by the inability of this method to distinguish between Pgp in normal versus neoplastic cells, demonstrating again the need for multiparameter analysis.

**Trial 4: Testing of Clinical Samples.** In this comparison, investigators used previously validated reagents and cell lines to analyze Pgp and MDR1 expression in specimens of AML, renal tumors, normal kidney and brain, and alveolar rhabdomyosarcomas.

**AML.** Table 3 compares the detection of Pgp expression in AML by flow cytometry and immunocytochemistry, using a panel of five anti-Pgp antibodies. The results obtained with the MRK-16 antibody, in contrast to the others, correlated best with dye-efflux data in these samples and thus represent the actual expression of Pgp. Flow cytometry was clearly superior in distinguishing specimens with low or intermediate levels of Pgp expression. In contrast to the spectrum of staining seen with MRK-16, identical results were produced by the C494, C219, and JSB-1 antibodies. The occasional false positive result yielded by immunohistochemical analysis with C219, C494, JSB-1, or 4E3 antibodies probably relates to the admixture of normal cells in AML specimens.

The correspondence of MDR1 mRNA detection in AML specimens by rt-PCR with the functional studies (Table 4) was not always clear,
Fig. 5. Trial 2: Immunohistochemical detection of Pgp in a solid tumor KB cell model. Cell buttons were prepared for embedding in paraffin by the sausage method illustrated in Fig. 4. Pgp density was quantitated by image analysis as detailed in "Materials and Methods."

because in some instances PCR identified negative samples as positive, perhaps because of the normal blood elements shown to express Pgp (55–57) in leukemic cell samples.

Solid Tumors. To assess the interlaboratory variability of Pgp determination in solid tumor specimens, the participating laboratories exchanged both snap-frozen and paraffin-embedded samples of normal kidney, renal cell carcinoma, and alveolar rhabdomyosarcoma, which were then fixed, air dried, and stained with anti-Pgp antibodies at the concentrations listed in Table 1. Staining of 80–90% of tumor cells in both frozen and paraffin-embedded sections of rhabdomyosarcoma was achieved with the C494 antibody (Fig. 7A); comparable results were obtained with C219. Variable staining was seen with 4E3, whereas HYB241 failed to identify specimens containing Pgp (Table 5).

Although the HYB241, C219, 4E3, UIC2, and MRK-16 antibodies uniformly stained normal kidney, mainly in the proximal tubules (Fig. 7B), they produced variable staining when applied to tissue partially effaced by renal carcinoma cells. For example, UIC2, 4E3, MRK-16, and...
MDR METHODS DETECTION WORKSHOP

Table 3 Comparison of flow cytometric and immunocytochemical detection of Pgp in AML samples using a five-antibody panel

<table>
<thead>
<tr>
<th>Method</th>
<th>AML patient</th>
<th>Antibody&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Method</th>
<th>AML patient</th>
<th>Antibody&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>4E3</td>
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<td></td>
<td>2</td>
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<tr>
<td></td>
<td>3</td>
<td>(14/1)</td>
<td>C219</td>
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<sup>a</sup> The data are summarized as the overall outcome of staining (+, positive results by >50% of the participating laboratories; -, <50% positive; ±, 50%). Numbers in parentheses denote the actual fraction of laboratories with a positive finding.

Results obtained with the MRK-16 antibody agree with dye-efflux functional studies and therefore represent the true variability of the different methods.

Table 4 Variability of MDRI/Pgp detection by rt-PCR and functional assays in identical samples of leukemia cells

<table>
<thead>
<tr>
<th>AML patient</th>
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<sup>b</sup> The data are presented as the overall study outcome for a particular assay (+, positive results by >50% of the participating laboratories; -, <50%; ±, 50%). Numbers in parentheses denote the actual fraction of laboratories achieving a certain result.

HYB241 failed to stain renal tumors altogether (Fig. 7B), whereas JSB-1 identified a few scattered cells that were regarded as background lymphocytes, macrophages, or both. By contrast, the C219 and C494 antibodies stained 80-90% of the tumor cells. Thus, recognition of Pgp expression in renal cell carcinoma may be difficult with immunohistochemical techniques. The variable results in this trial likely reflect the dependence of antibody sensitivity on fixation conditions and other factors, as shown in trial 2.

CONSENSUS RECOMMENDATIONS

The major goals of this workshop were to determine the inter- and intrastitutional variability of measurements of Pgp and MDRI levels in clinical specimens, to form a working group of investigators committed to improving the quality of research in MDR, and to establish guidelines for the detection of Pgp and MDRI in clinical specimens. We believe that all of these objectives were met.

The following are consensus recommendations for the detection of MDRI/Pgp expression and Pgp function in clinical specimens, arrived at by analysis of results in the workshop trials. It will be seen that many of the statements on immunohistochemical analysis, below, are the same as those that follow for flow cytometry. This duplication was considered necessary because of readers who might be searching for information on specific methods. An outline form of presentation was selected to enhance information retrieval.

I. Detection of Pgp by ICC/IHC

A. Overall Assessment. ICC/IHC has intrinsic advantages for the detection of Pgp expression in leukemias and solid tumors, including: (a) delineation of microanatomic details; (b) recognition of tumor cell heterogeneity; (c) delineation of admixed normal cells bearing Pgp; and (d) opportunity to correlate histology and phenotype. Subjectivity is the primary disadvantage.

B. Antibody Reagents. Because some commercially available anti-Pgp antibodies recognize epitopes on other molecules (21, 22), at least two antibodies recognizing separate epitopes (one surface, one cytoplasmic) should be used; only limited confidence can be placed in studies using single antibodies (6).

C. Quality Control. Antibody manufacturers are strongly encouraged to control lot-to-lot variability in their analytic reagents, to

A. Rhabdomyosarcoma

**C494**

**Paraffin section with C494**

**Negative Control without C494**

**Normal Kidney Control**

**Renal Carcinoma Control**

B. Renal Carcinoma

**UIC2**

**Paraffin section with UIC2**

**Negative Control without UIC2**

**Normal Kidney Control**

**Renal Carcinoma Control**

Fig. 7. Trial 4: Detection of Pgp by immunohistochemical staining in solid tumors and normal kidney. Staining of Pgp with antibodies C494 and UIC2 are shown in representative paraffin-embedded sections of rhabdomyosarcoma, normal kidney, and renal carcinoma. See “Materials and Methods” and text for further details.
provide information on antibody purity and specificity, and to standardize their preparations of anti-Pgp antibodies with well-characterized MDR1/Pgp-expressing cell lines, as suggested by the workshop. (Also see sections II.B.1 and II.B.2, below.)

D. Controls. Well-characterized MDR1/Pgp-expressing cell lines, antibody specificities, and fixation-control tissues should be matched to the specific tumors and organ sites under investigation. It is critical that cell lines reflect the physiological or pathophysiological level and pattern of site-specific Pgp expression in vivo. For example, we favor the 8226/Dox cell line over the 8226/Dox40 line for studies of myeloma and other hematological malignancies, in part because its level of drug resistance and Pgp expression is lower and more clinically relevant. Finally, a “gold standard” control cell line should be used in multiparameter MDR1/Pgp analyses. Although the 8226/Dox series is well suited for this purpose (27), other well-characterized Pgp-expressing cell lines, such as the CEM/VLB series of increasing but low levels of resistance, would also be appropriate (e.g., Refs. 25, 26, and 45).

E. Standardization of Tissue Handling and Preparation

1. Cytospin Preparations. We recommend cytospin preparations of cells freshly dipped in ice-cold (4°C) acetone for 10 min after air drying. The slides should then be stored in a cool, dry environment until the time of assay.

2. Preparations of Snap-Frozen Sections. We recommend that freshly acquired tissue samples be embedded in OCT compound and snap-frozen in liquid nitrogen alone or in isopentane (at —150°C) and then air dried. The slides should then be stored in a cool, dry environment until the time of assay.

3. Paraffin-embedded Tissues. Standardized fixation should be used with paraffin-embedded tissues in prospective studies. We recognize the limitations of data interpretation in retrospective analyses based on paraffin-embedded tissues sections; hence, the conditions for optimization of Pgp detection are important and will be reported in detail elsewhere. In general, we recommend a brief (4 h) tissue fixation in 10% neutral buffered formalin for Pgp assays not requiring antigen retrieval. Antigen retrieval methods are required when longer fixation periods and other fixatives are used.

F. Data Analysis. Distinguishing between Pgp expression in tumor versus normal cells (e.g., Refs. 55–57) is a major challenge one faces when analyzing the results of immunohistochemical assays. Moreover, the relative importance of the level of Pgp expression (i.e., intensity of antibody staining) and the percentage of Pgp+ cells is currently unknown. We therefore recommend that both types of data be collected in any study of Pgp expression.

1. The intensity of antibody staining should be reported as negative (−), low (+), intermediate (++), high (+++), or ultrahigh (++++)

2. We strongly recommend that arbitrary minimal cutoff points not be used in data analysis.

G. Multiparameter Assays. All workshop participants encouraged the use of multiparameter assays in studies of tumor cell Pgp expression. Ideally, the methods should include assessments of protein level (immunocytochemistry or immunoblot analysis), mRNA (RT-PCR or RNase protection assay), and functional activity (drug or dye efflux). Pgp+ cell lines, such as 8226/Dox or CEM/VLB, should be used as standards to validate methods and control for variability.

II. Flow Cytometric Detection of Pgp Expression. Most of these comments refer to the analysis of Pgp and MDR1 in hematological specimens (bone marrow, blood, and disaggregated lymph nodes). Evaluation of solid tumors, although addressed, was not a major topic in this workshop because of the special problems posed by analysis of Pgp expression in such tissue. Nonetheless, some of the following recommendations also apply to specimens of disaggregated solid tumors, as noted.

A. Sample Handling. Flow cytometric assessment of Pgp should be performed on fresh samples; on transported samples that have been held for no longer than 24 h, either at room temperature or on wet ice; or on samples that have been cryopreserved as cell suspensions frozen in 20–90% fetal bovine serum and 10% DMSO at −135°C (15). These methods of sample handling also allow mRNA to be isolated for analysis of MDR1 by RT-PCR.

B. Antibody Reagents

1. Epitopes. For flow cytometric studies, anti-Pgp antibodies that have been shown to be specific for Pgp and that recognize external epitopes are recommended. These presently include MRK16 (33), UIC2 (32), 4E3 (29), and HYB241 (34). Whereas MRK16 and UIC2 can detect Pgp in the majority of leukemia cases that are positive for MDR1 by RT-PCR, HYB241 may only recognize this protein in a subset of MRK+ cases of myeloid leukemia (Ref. 15 and references therein). Anti-Pgp antibodies that recognize external epitopes are preferred because they allow correlation of Pgp expression with other cell surface antigens by multicolor flow cytometry and with functional measurements of dye efflux/accumulation. They also allow analysis of Pgp expression on leukemic cells (see section II.D (Gating), below) with exclusion of residual normal Pgp+ cells (e.g., T cells, CD56+ cells) and dead cells that have taken up antibody non-specifically.

2. Quality Control. Antibodies used for Pgp detection must be rigorously controlled for quality. We recommend that commercial vendors affinity-purify Pgp-specific antibodies; test antibody purity by SDS-PAGE or other suitable methods; and test antibody specificity in ELISA assays, as well as on a series of standardized drug-sensitive and increasingly drug-resistant Pgp-expressing cell lines, such as the 8226/Dox series (27), the CEM/VLB series (25), or the KB-series (26, 45). We strongly recommend that manufacturers provide complete standardization information with each new lot of antibodies, and we encourage the development of new high-affinity anti-Pgp antibodies.
3. Antibody Staining and Fluorochrome Detection. Anti-Pgp antibodies should be directly conjugated to either a fluorochrome (e.g., PE; Refs. 15 and 58) or the duochrome reagent streptavidin-Texas Red-PE (15, 58) rather than to FITC. Detection with a secondary antibody conjugated to a red fluorochrome instead of to FITC is preferred. PE and the duochrome reagent are regarded as superior for at least two reasons (58): (a) the former reagent has a much higher quantum efficiency than FITC and permits more reliable detection of low but significant levels of Pgp expression in hematological samples (58). Indeed, some participating investigators reported that a fraction of leukemic samples were Pgp+ with use of a PE conjugate but negative with FITC conjugates, particularly cells with low Pgp antigen density; and (b) because normal cells autofluoresce in the blue-green (FITC) range (58), use of dyes that emit in the red allows detection of a wider range of channel shifts above the background control (58), thereby permitting greater S:N ratios and allowing for detection of cells with very low Pgp expression.

C. Controls
1. All antibody reactions should have an isotypically matched control, used at the same concentration as the primary anti-Pgp antibody. A "no-primary-antibody" control is also required.
2. As recommended for immunocytochemical staining, well-characterized Pgp-expressing cell lines are required to validate and standardize flow cytometric assays and to calibrate instruments. Workshop participants recommended the use of the low-level Pgp-expressing doxorubicin-resistant series of 8226 myeloma cell lines (27), although, as discussed above, any well-characterized cell line series of low Pgp expression (e.g., some CEM/VLB sublines; Ref. 25) can be used. Because of the very low Pgp levels in most leukemic samples, compared to those in Dox6 cells, participants recommended the development of more Pgp-expressing sublines with resistance levels between those of 8226/S and Dox6. Participants suggested, moreover, that standardized Pgp-MDR cell lines be maintained and distributed from a single reference laboratory or company.

D. Gating. Electronic windows (gating) should be used to analyze Pgp expression by cells with the size and granularity of blast cells. This method allows selective study of Pgp expression on leukemia/lymphoma cells and eliminates residual normal cells that express Pgp+, a potentially significant problem in quantifying Pgp expression (15).

E. Data Analysis. There are many ways to evaluate and report flow cytometric data, including the percentage of positive cells, assessment of the mean channel shift (difference in mean channel between the control and the experimental sample), and the D value (Kolmogorov-Smirnov statistic; Ref. 15). Each of these measurements has its strengths and weaknesses (15), precluding recommendation of a preferred method; however, the participants agreed to report data as continuous variables rather than establish a cutoff point for assessing positivity.

III. Measurement of Pgp Function
A. Dye or Drug Efflux.
1. Measuring dye or drug efflux in the presence and absence of a Pgp modulator (e.g., cyclosporin, PSC388, or verapamil) is preferable to measuring dye or drug accumulation only (15, 44–46).
2. Several dyes (DIOC2, Rhod23, or Hoechst) or drugs (daunorubicin or doxorubicin) may be used to assess Pgp function (15, 45, 46); however, some dyes appear to yield better functional estimates than do the actual drug substrates (15), probably because of more favorable uptake/efflux kinetics. Because the basis for this discrepancy remains unclear, we recommend that both methods be applied to clinical specimens.

3. Because most functional measurements are performed with flow cytometry, it is desirable to correlate the functional parameter (dye efflux) with Pgp protein expression as detected with anti-Pgp antibodies. With this approach, it is possible to detect drug efflux in the absence of Pgp expression, an indication of other (non-MDR) mechanisms of drug resistance (45).

B. Amplification Reaction. All rt-PCR assays should be performed in the exponential range and not the plateau range of the amplification reaction. This allows detection of MDR1 expression in the dominant cell population of a sample, rather than in rare subpopulations. Because competition can occur between different primer sets in a multiplex reaction, it is essential to show no competition between primer pairs.

C. Internal Standards. Both β-2 microglobulin (50) and histone 3.3 (49) afford reliable internal standards to ensure that samples contain high-quality RNA suitable for PCR amplification. We do not recommend the use of β-actin, as its expression is variable and can decrease in drug-resistant cells (59).

D. Controls. As with flow cytometry, it is essential to establish PCR assays with negative controls and with a standard series of drug-sensitive and increasingly resistant cell lines (see section II.C).

FUTURE CONSIDERATIONS
One of the most important achievements of the St. Jude workshop was to reach consensus conclusions on means to standardize methods for the study of Pgp expression in clinical material. It was also clear that hematological malignancies offer the best current opportunity for reliable detection of this protein, and that much work remains to be done before solid tumors can be profitably examined for Pgp overexpression. Given the complexity of solid tumors, in which clinical drug resistance can have pharmacodynamic and pharmacokinetic elements, as well as biochemical elements, any future workshop on MDR must focus on the detection of Pgp in these types of neoplasms. It will also be important to expand the workshop agenda to include other markers of MDR, such as MRP (45; 60–63), LRP (64, 65), topoisomerase II (66), and others. Although multiparameter analyses are clearly superior in studies of Pgp levels and function, there is still a need to develop novel strategies that would integrate the specificity of molecular methods with methods of cellular delineation (e.g., immuno-PCR or immuno-in situ, or in situ PCR) to improve the identification of resistance-associated proteins.

Many unresolved questions cannot be addressed with confidence until gene/protein detection methods and the understanding of resistance mechanisms have improved. For example, although we believe that we can now reliably measure Pgp, at least in hematological malignancies, accurate measurement of Pgp expression under most conditions remains an elusive goal. Indeed, it is still not clear what methods are most appropriate for determining the MDR status of patients’ tumors. Clarification of this issue should allow determination of the numbers of Pgp molecules (or other markers of drug resistance) required to confer resistance to the tumor or tumor cell. This is directly related to our present ignorance about the level of, e.g., Pgp detection that correlates with the acquisition of drug resistance by a patient’s tumor cells. Once a consensus regarding the “truth” of Pgp
expression in a tumor has been reached, it will be possible to improve the design of retrospective and prospective studies of how MDR1 expression is related to clinical outcome. Another critical question is whether or not discrepancies among different Pgp/MDR1 assays reflect intrinsic differences in basic tumor cell biology. Our hope is that these and other issues will be topics at future MDR workshops attended by laboratories worldwide.

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REFERENCES


MDR METHODS DETECTION WORKSHOP


Methods to Detect P-Glycoprotein-associated Multidrug Resistance in Patients' Tumors: Consensus Recommendations


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