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Original Paper

Examination of Multidrug Resistance in Cell Lines and Primary Breast Tumours by Flow Cytometry*

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The aim of this study was to measure multidrug resistance (MDR) by flow cytometry and quantify the expression of P-glycoprotein (using antibody) glutathione transferase (using α-GSTpi antibody) in α-JSB-1 and α-GSTpi of a series of cell lines and primary breast cancers, and to assess the relationship between these MDR proteins and a selection of oncogene and prognostic markers in breast cancer. Flow cytometry was performed using permeabilised cells stained with fluorescent antibodies using well-established methods. Antibody staining was confirmed for JSB1, but not GSTpi by use of known positive and negative controls. No correlation was seen when comparing the number of molecules of α-JSB-1 with α-GSTpi ($P = 0.1$, $r^2 = 0.4$, $n = 14$) using a selection of cell lines. Examination of 45 breast tumours for expression of JSB-1 and GSTpi revealed a significant association between these two antibodies ($P < 0.00001$, $r^2 = 0.5$, $n = 45$). On examining the breast tumours, α-JSB-1 showed a positive association with c-erbB-2 ($P = 0.003$), c-myc ($P = 0.0004$) and c-jun ($P = 0.02$) but not ER or EGF-R expression. α-GSTpi showed a positive association with c-erbB-2 ($P = 0.03$) and c-myc ($P = 0.0004$) but not ER, EGF-R or c-jun. Flow cytometric MDR levels were not related to tumour grade or axillary node status. In solid tumours, a relationship between the two antibodies used has been clearly demonstrated, however, specificity of α-GSTpi is questioned. Both antibodies show an association with c-erbB-2, which is associated with poor prognosis and with c-myc which is involved in cell cycling and differentiation. Monitoring MDR markers (Pgp) using this methodology may be useful for evaluation of prognosis in breast cancer. Copyright © 1996 Elsevier Science Ltd

Key words: flow cytometry, multidrug resistance, breast, cancer

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INTRODUCTION

ONE OF the main reasons that chemotherapeutic drugs fail to eliminate tumours completely is the development of multidrug resistance (MDR). MDR involves tumours becoming resistant to a wide spectrum of non-related drugs (anthracyclines, vinca alkaloids and podophylotoxins) either *de novo* or following chemotherapeutic treatment [1].

Increased expression of the *MDR1* gene leads to increased steady state levels of a high molecular weight (170 kDa) membrane P-glycoprotein (Pgp or P170). [2] P-glycoprotein has been found in plasma membranes and on the luminal side of Golgi stacks [3] and is believed to act as an efflux pump [4, 5] expelling drugs across the cell membrane [6] or relocalising the drug into subcellular organelles [7]. The efflux system is dependant on calcium channels and may be blocked by addition of drugs such as verapamil, which bind to Pgp and allow cytotoxic drug accumulation [8]. In studies of cell lines, increased expression of Pgp correlates with decreased accumulation and retention of drugs intracellu-

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larly [4]. Expression of MDR has been shown to correlate with clinical drug resistance in pretreated haematological cancers [2] and to occur prior to exposure to cytotoxic agents in breast cancer [9]. Immunohistochemical examination of Pgp expression in locally advanced breast cancers has been performed using several monoclonal antibodies including C219 [10] and JSB-1 [11]. JSB-1 was reported to show excellent immunostaining of cytological preparations [11]. It was further noted that some degree of permeabilisation was a prerequisite for exposing the epitope recognised by JSB-1. This finding lends itself to flow cytometric methodologies used previously by us [12, 13].

Further mechanisms of MDR have been described and include decreased expression and changes in the catalytic activity of the enzyme topoisomerase II and alteration in glutathione transferase levels. Glutathione has the ability to scavenge free radicals, and increased levels of the enzyme for this reaction, glutathione-S-transferase, may increase the metabolism of cytotoxic and chemotherapeutic drugs [14]. Glutathione-S-transferase (GST) exists in three main isoforms, neutral, basic and acidic which are expressed in varying degrees by most tumours. In invasive breast carcinoma, the acidic form has been shown to produce intense nuclear and cytoplasmic staining patterns with the antibody NCL-GSTpi immunohistochemically [15].

Methods for the detection of MDR are wide and results are varied [16–18]. Measurement of MDR by flow cytometry has concentrated on examination of antibodies to Pgp [20] [20] and on assays to measure glutathione levels [21]. Several problems exist in the measuring of MDR, not least the fact that both Pgp and GST are expressed by 'normal' cells, which are likely to be present in any heterogeneous primary breast tumour population sampled. Furthermore, clinically relevant cut-off levels for MDR are not available and low levels of native antigen expression have been cited as reasons for some of the methodological problems [19].

As the overexpression of *MDR1* has been associated with clinical drug resistance in a number of malignancies, monitoring of markers of MDR may prove important as an indicator of the success of drugs designed to circumvent the development of clinical multidrug resistance. We have attempted to measure MDR by flow cytometry and quantify the expression of two antibodies, α-JSB-1 directed against Pgp and α-GSTpi (placental/acidic) directed against glutathione-S-transferase, on a series of cell lines and primary breast cancers. We have further assessed the relationship between these MDR markers and the oncogene markers c-erbB-2, c-myc, c-jun and the prognostic markers oestrogen receptor (ER), epidermal growth factor receptor (EGF-R), Bloom and Richardson tumour grade and axillary node status in breast cancer.

MATERIALS AND METHODS

Patient case series

Samples of solid breast tumours were obtained from 45 consecutive women undergoing mastectomy for diagnosed primary breast cancer. Routine histopathology was performed with Bloom and Richardson grade, tumour size and axillary node status recorded. Remaining material for flow cytometric analysis was stored in liquid nitrogen prior to analysis.

Cell line preparation

Mycoplasma screened adherent breast tumour cell lines T47, HBL100, MCF7, SKBR3, MDA, MDA231, EFF3, SVK, the adherent cervical tumour cell line HeLa (Department Pathology, RVI, Newcastle upon Tyne, U.K.), the lung epithelial cell line A549, and MDR sensitive and resistant clones of the KK47 bladder cell line (J. Lunec, Department of Cancer Research, University Newcastle upon Tyne, U.K.) were cultured in DMEM (Northumbria Biologicals, Cramlington, Northumberland, U.K.) supplemented with 10% fetal calf serum (GIBCO, Paisley, Glasgow, U.K.), 50 U/ml penicillin/streptomycin (Northumbria Biologicals) and 20 mM, L-glutamine (Northumbria Biologicals). Adherent cells were harvested by brief treatment with 10 ml 1 × trypsin–EDTA solution (0.05% trypsin/0.02% EDTA, Northumbria Biologicals). Harvested cells were washed in 10 ml Isoton II (Coulter Electronics Ltd, Bedfordshire, U.K.) before processing for MDR measurement using the monoclonal NCL-JSB-1 (anti Pgp) and polyclonal NCL-GSTpi antibodies (Novocastra Laboratories Ltd, Newcastle upon Tyne, U.K.).

Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC, negative control) were isolated from heparinised peripheral blood donated by normal, healthy adult volunteers as previously described [12].

Preparation of primary breast tumour and liver samples

Solid tumour samples were obtained on the day of operation from patients undergoing elective mastectomy for breast cancer. Liver specimens (positive stain control) were obtained from pathological specimens. Solid tissue samples were rapidly thawed, finely minced and further disaggregated by passing through a fine wire mesh (approximately 50 μm) to form a single cell suspension in Isoton II (Coulter).

Flow cytometry

Cell lines. The suspensions of lymphocytes, liver and tumour cell lines (approximately 5×10^6 cells/ml Isoton II) were aliquoted into 50 μl samples in LP10 tubes (S.H. Scientific, Northumberland, U.K.). To each sample, 50 μl of 1% saponin (BDH, Poole, Dorset, U.K., in Isoton II) or 50 μl of Isoton II (Coulter) were added with gentle mixing and the mixture was allowed to stand at room temperature for 2 min. A saturating concentration of 10 μl of α-NCL-JSB-1 (for Pgp detection) or α-NCL-GSTpi (for GST detection) antibodies (Novocastra) were then added to each cell type to be tested. Further samples of each cell suspension were stained with 5 μl (equivalent concentration compared with test) of an appropriate isotype control. All samples were incubated at 4°C for 20 min and then washed with Isoton II (Coulter) using a preprogrammed cell wash cycle (Ross Labs, Cheshire, U.K.). The cell pellet was resuspended in 100 μl of goat antimouse FITC (JSB-1 stained, BD, Oxford, U.K.) or 3 μl sheep antirabbit FITC conjugated antibody (Sigma, Dorset U.K.). All samples were incubated at 4°C for 20 min and then washed with Isoton II (Coulter) using a preprogrammed cell wash cycle (Ross Labs). The cell pellet was resuspended in 0.5 ml Isoton II and flow cytometry was performed on a FACScan

flow cytometer (BD). Excitation was produced by an argon laser (15 mW) using the following prestored compensated settings: forward scatter (detector E00, LIN amplifier 1.38 V), side scatter (detector 288 V, LIN amplifier 2.30 V), FL1 (4 decades, detector 620 V, LOG amplifier 4.25 V, fluorescence compensation 0.7%). Data analysis was performed using Lysis II software with cells gated on a dot plot of FSC versus SSC. Median fluorescence (FITC) values were determined from the FL1 histogram for unstained (isotype control) and stained cells. Isotype control median values were subtracted from the positive stained median and binding capacities were evaluated from the standardised QSC bead equation.

Solid breast tumour samples. The suspensions of primary tumour cells (approximately 1×10^6 cells/ml Isoton II, $n = 45$) were prepared and stained for MDR expression as for cell lines. To other samples of these tumours, 10 µl of cytokeratin-5D3 (CK-5D3) FITC (Novocastra Laboratories Ltd, Newcastle upon Tyne, U.K.) alone or in conjunction with 2.5 µl of biotin conjugated anti-c-erbB-2 ($n = 35$), anti-EGF-R ($n = 32$), anti-ER ($n = 34$), c-myc ($n = 25$) and c-jun ($n = 22$) antibody (Novocastra) were then added to each tumour cell suspension to be tested. (Note: the selection of tumours for each stain was random and not influenced by pathological or physical tumour properties.)

Another sample was stained with 10 µl of GSTpi (Rabbit polyclonal antibody, Novocastra) followed by 3 µl sheep antirabbit FITC conjugated antibody (Sigma, Dorset U.K.) after a primary incubation as per JSB-1 stained cells.

Other samples of each cell suspension were stained with 5 µl IgG-2b-FITC isotype control (Coulter) or with 10 µl of streptavidin PE (BD). All samples were incubated at 4°C for 20 min and then washed with Isoton II containing 1% saponin. To those cells labelled with biotinylated antibody, 10 µl PE conjugated streptavidin (BD) antibody was added to the cell pellet as previously described. After incubation and washing, the cell pellet was resuspended in 0.5 ml Isoton II and flow cytometry was performed as previously described. Data analysis was performed using Lysis II software with cells gated on a dot plot of FSC versus SSC. Cytokeratin positive cells were backgated to define the FSC versus SSC gate, which contained the greatest percentage of cytokeratin positive cells. As SSC and FSC remain the same for each tumour, we could decrease the number of cytokeratin negative cells examined in those cases where cytokeratin antibody was not used i.e. MDR staining. Geometric median fluorescence (PE) values were determined from the FL2 histogram for SPE stained (control) and biotinylated antibody stained cells. Binding capacities were evaluated from the standardised QSC bead equation.

Antibody standardisation by flow cytometry. All antibodies and secondary stains (except the polyclonal antibody α-NCL-GSTpi) were incubated with 50 µl of Quantum Simply Cellular bead standards (QSC, Flow Cytometry Standards corporation, North Carolina, U.S.A.) at 4°C for 30 min as previously described [12, 13]. GSTpi was calibrated using FCSC beads labelled with fluorescein (flow cytometry standards).

RESULTS

In order to determine the specificity of JSB-1 and GSTpi, screening was performed on a series of sensitive, multidrug resistant and unknown tissues and cell lines. This panel included the known multidrug resistant and sensitive clones of the KK47 cell line. Liver, which shows intense staining of bile ducts with GSTpi and bile canalicular staining with JSB-1 immunohistochemically, was included as a positive control and as an indicator of high fluorescence staining levels in non-cancerous material. Due to their low-level staining pattern, lymphocytes were included as a negative, non-malignant cell control and as an indicator of the fluorescence staining threshold.

Binding capacities were determined for each cell line or tissue using calibration curves constructed from bead standards. Due to the fact that neither JSB-1 nor GSTpi antibodies were available in a conjugated form and GSTpi was a rabbit antihuman polyclonal antibody, the scope for multiparametric analysis was limited. Variations in the usual calibration methodology were necessary. Although somewhat unsatisfactory, it was necessary to use fluorescently labelled beads to calibrate GSTpi expression. These beads do not give a true value for the number of molecules expressed due to the inability to correct for the *f/p* (number of fluorescence molecules/protein) ratio of the SAR FITC antibody. However, this could be corrected for in the case of NCL-JSB-1 which was a mouse antihuman monoclonal antibody as the beads used are of known binding site number. The lack of enough antibody constrained our use of both JSB-1 and GSTpi antibodies in combination with cytokeratin. For this reason, histological screening for tissue areas containing high numbers of tumour cells was performed. Solid tumours demonstrated high levels of cytokeratin positive cells in all cases with around 90% of cells in each tumour staining positively for cytokeratin with minimal contamination by non-epithelial cells.

Figure 1a illustrates the linear relationship between increasing fluorescence channel number and JSB-1 binding capacity for a range of cell lines and fresh tissues. Lymphocytes provided a good negative control with a value of 2582 molecules/cell. MDA (4094 molecules/cell), EFF3 (3971 molecules/cell) and MCF7 (4448 molecules/cell) cell lines showed low expression of JSB-1, while the multidrug sensitive cell line MDS (KK47) showed a low binding capacity (3029 molecules/cell), but slightly higher fluorescence channel number. This was associated with non-specific staining. Correction of this non-specific staining by subtraction of the MDS isotype control level was reflected in the low binding capacity illustrated in Figure 1a. Mid-range expression of JSB-1 was shown by HeLa (7405 molecules/cell), A549 (6804 molecules/cell), MDA231 (8576 molecules/cell) cell lines. High levels of expression of JSB-1 were shown by the T47 (11 252 molecules/cell), HBL100 (11 595 molecules/cell), SKBR3 (12 567 molecules/cell), SVK (14 982 molecules/cell) cell lines, the positive control liver tissue (12 978 molecules/cell) and the known multidrug resistant clone of the KK47 cell line (MDR, 14 975 molecules/cell).

Figure 1b illustrates the linear relationship between increasing fluorescence channel number and GSTpi binding capacity for a range of cell lines and fresh tissues. Lymphocytes again provided a good negative control with 1500 molecules/cell, a similar threshold level to that of the JSB-1 antibody. MDA (14 280 molecules/cell), EFF3 (10 314 molecules/cell) and MCF7 (20 069 molecules/cell)

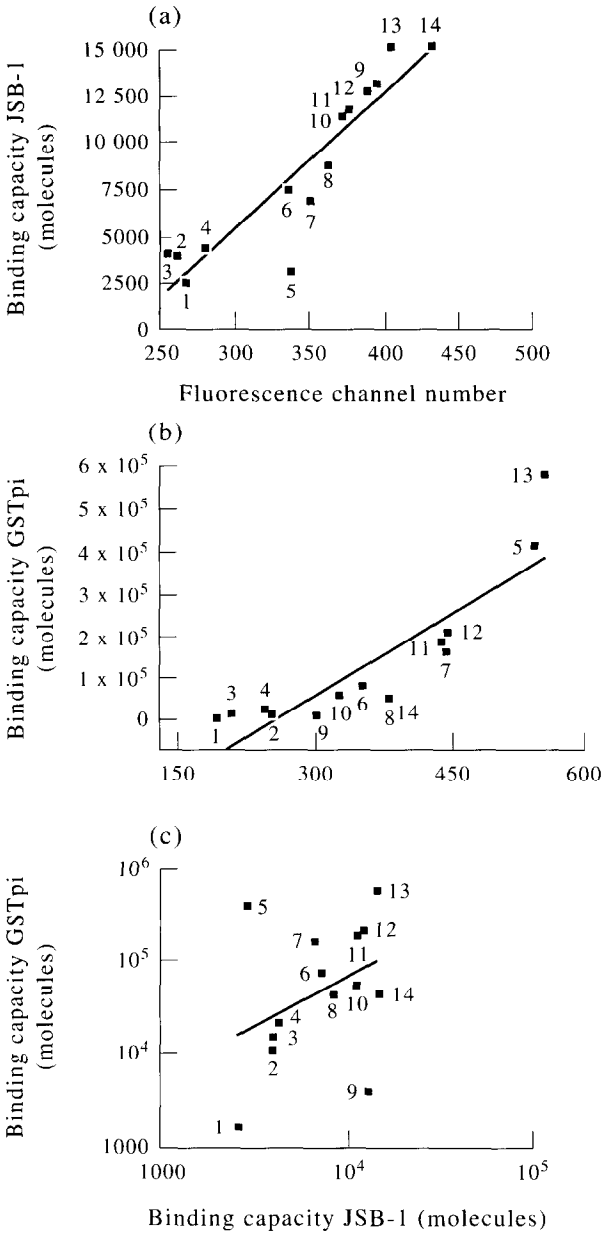

Figure 1. The binding capacity (number of molecules) of (a) JSB-1 and of (b) GSTpi on a variety of cell lines. (c) The relationship between JSB-1 and GSTpi on these cell lines. Cell lines are numbered: 1, Lymphocytes; 2, EFF3; 3, MDA; 4, MCF7; 5, KK47 sensitive (MDS); 6, HeLa; 7, A549; 8, MDA231; 9, Liver; 10, T47; 11, HBL100; 12, SKBR3; 13, KK47 resistant MDR; 14, SVK.

cell lines showed low expression of GSTpi when taken as a whole. The number of molecules of GSTpi was higher than those of JSB-1 and reflects the inability to correct for the *f/p* ratio. Moderate expression of GSTpi was shown by HeLa (69 777 molecules/cell), T47 (49 515 molecules/cell), SVK (40 414 molecules/cell) and MDA231 (38 688 molecules/cell) cell lines. Very high levels of expression of GSTpi were shown by the A549 (148 262 molecules/cell), HBL100 (169 339 molecules/cell) and SKBR3 (191 100 molecules/cell) cell lines. Unexpectedly, both clones of the KK47 (MDR 560 581 molecules/cell, MDS 393 403 molecules/cell) cell line showed extremely high levels of GSTpi expression. The positive control, liver, showed a low level of staining (3443 molecules/cell), despite repeated attempts with a variety of liver samples. The GSTpi results cast some doubt upon the efficacy of this antibody for use by flow cytometry. These discrepancies in expected staining pattern are indicative of a non-specific staining pattern.

On comparing the number of molecules of JSB-1 with the number of molecules of GSTpi on each cell line, no significant correlation was found ($P = 0.1$, $r^2 = 0.4$, $n = 14$). However, in the absence of the MDS point and positive liver control point, significance was greatly increased ($P = 0.006$, $r^2 = 0.52$, $n = 12$, Figure 1c). Axes were plotted using logarithmic scales in order to prevent clustering of data at either very low or very high levels. The unexpected results were consistent despite repeating the assay and may reflect a serious problem of specificity of the GSTpi antibody.

With these findings noted, we applied the technique to solid primary breast tumours. Forty-five paired, solid, breast tumour samples were stained for JSB-1 and GSTpi expression. Figure 2 illustrates the relationship between JSB-1 and GSTpi expression for 45 paired, primary, breast cancers. Regression analysis revealed a significant association between expression of both antibodies ($P < 0.00001$, $r^2 = 0.5$, $n = 45$). In solid breast tumours, raised levels of Pgp were associated with raised levels of glutathione-S-transferase. Levels of JSB-1 expression are much higher in the primary breast tumour group compared with cell lines. GSTpi expression tended to show the reverse.

Thirty-seven of the 45 breast cancers collected were given a Bloom & Richardson grading. Of these, two were classified grade I, 18 grade II and 17 grade III. The remaining eight samples were identified as ductal carcinoma *in situ* (DCIS, $n = 6$) or were unclassified (lobular carcinomas, $n = 2$).

Examination of JSB-1 or GSTpi expression and Bloom & Richardson grade was performed. Using Mann Whitney analysis, no significant difference was seen between grade I and grade II tumour expression of JSB-1 or GSTpi ($P = 0.9$, $P = 0.8$). Similarly, on comparison of grade I and grade III ($P = 0.9$, $P = 0.6$) and grade II and grade III

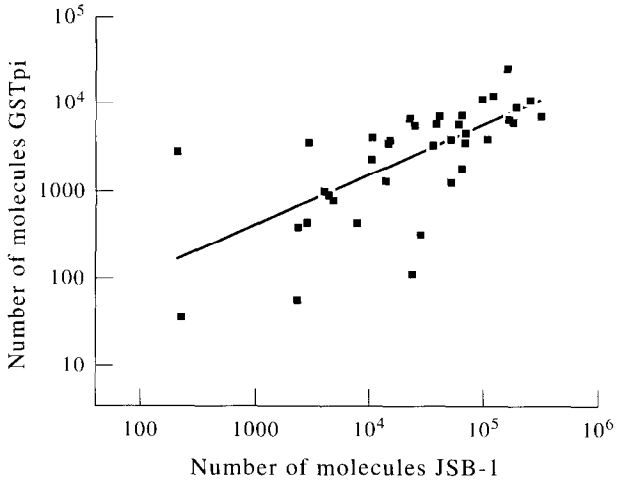

Figure 2. The relationship between JSB-1 and GSTpi for 45 primary breast tumour samples. A strong correlation was observed ($P < 0.00001$, $n = 45$).

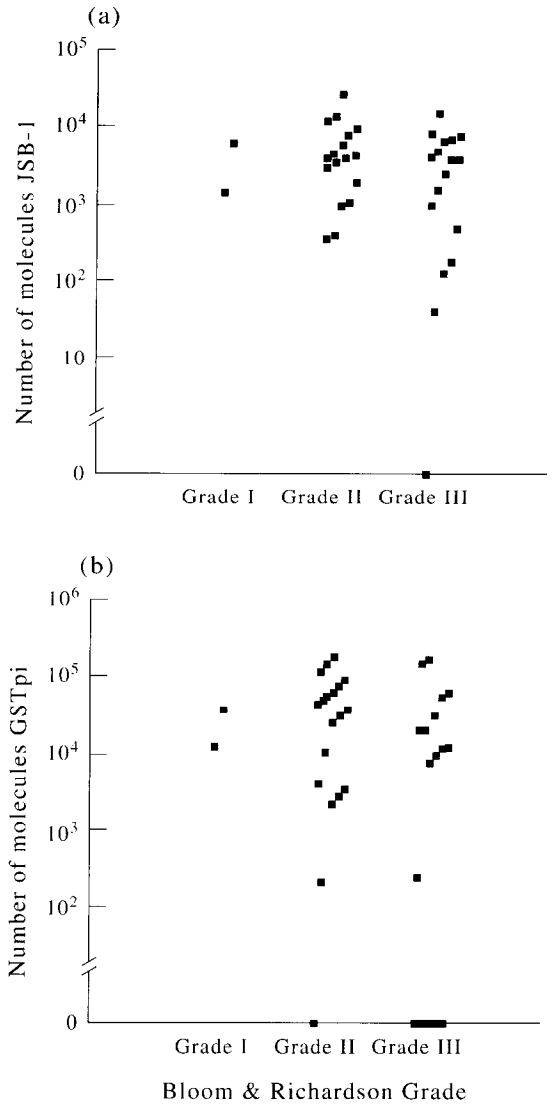

Figure 3. The expression of (a) JSB-1 and (b) GSTpi in primary breast tumours categorised by Bloom & Richardson grade. No significant differences were seen for either antibody with grade.

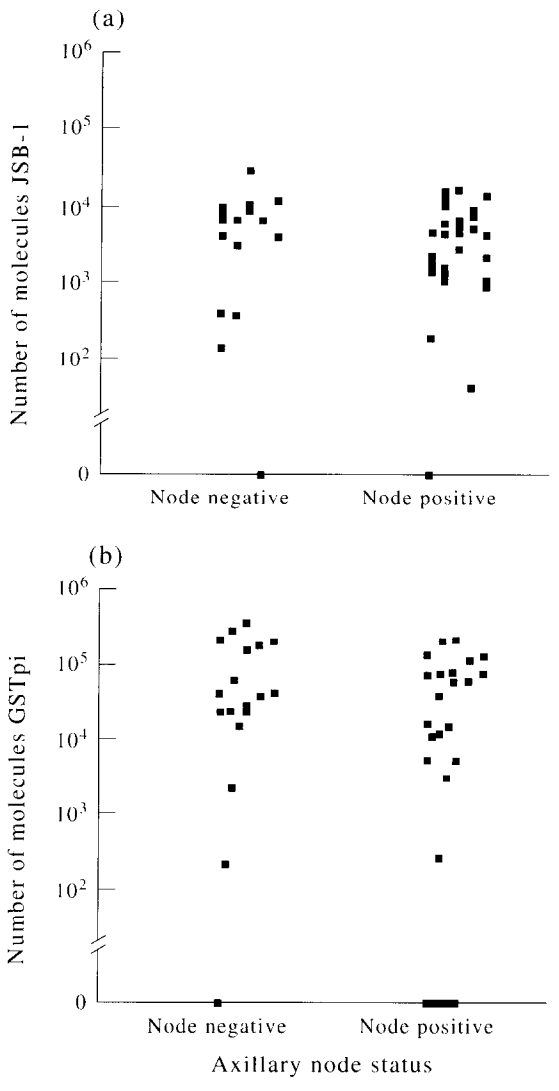

Figure 4. The expression of (a) JSB-1 and (b) GSTpi in primary breast tumours categorised by axillary node status. No significant differences were seen between node-negative and node-positive groups for either antibody.

($P = 0.7$, $P = 0.2$), no significant differences in the level of JSB-1 or GSTpi, respectively, was seen (Figure 3a,b).

All 45 patients were examined for axillary node status and data was available for each of the 45 tumours stained for expression of JSB-1 or GSTpi. Using Mann Whitney analysis, no significant difference was seen between JSB-1 expression in axillary node-negative compared with axillary node-positive patients ($P = 0.2$, Figure 4a). A similar finding was shown for GSTpi expression ($P = 0.09$, Figure 4b).

Comparison of JSB-1 levels against cytokeratin gated cells stained for EGF-R ($P = 0.2$, $n = 32$) and ER ($P = 0.7$, $n = 34$) showed no correlation. However, c-erbB-2 (Figure 5a, $P = 0.003$, $n = 35$), c-myc (Figure 5b, $P = 0.0004$, $n = 25$) and c-jun (Figure 5c, $P = 0.02$, $n = 22$) did show a significant correlation.

Comparison of GSTpi levels against cytokeratin gated cells stained for EGF-R ($P = 0.5$, $n = 32$), ER ($P = 0.8$, $n = 34$) and jun ($P = 0.09$ $n = 22$) showed no correlation. However, c-erbB-2 (Figure 6a, $P = 0.03$, $n = 35$) and c-myc (Figure 6b, $P = 0.0004$, $n = 25$) did show a significant correlation. It was noted that the correlations of JSB-1 and GSTpi staining with c-myc may be effected by three strongly staining samples.

DISCUSSION

The question proposed by Dalton [17] and faced by the clinician concerned with multidrug resistance is, "Is multidrug resistance, due to the overexpression of *MDR1* P-glycoprotein, an important factor in determining therapeutic outcome?" In order to determine the answer to this question, expression of potential markers of MDR must be assessed in solid human tumours. Flow cytometry is one method which allows examination of phenotype in an individual cell. In the heterogeneous population of cells within a solid tumour, it may be important to examine individual phenotypes. Ideally, we would like to examine MDR expression in conjunction with cytokeratin expression. However, the value of cytokeratin gating [13, 22, 23] is primarily important in the analysis of FNA samples and in the

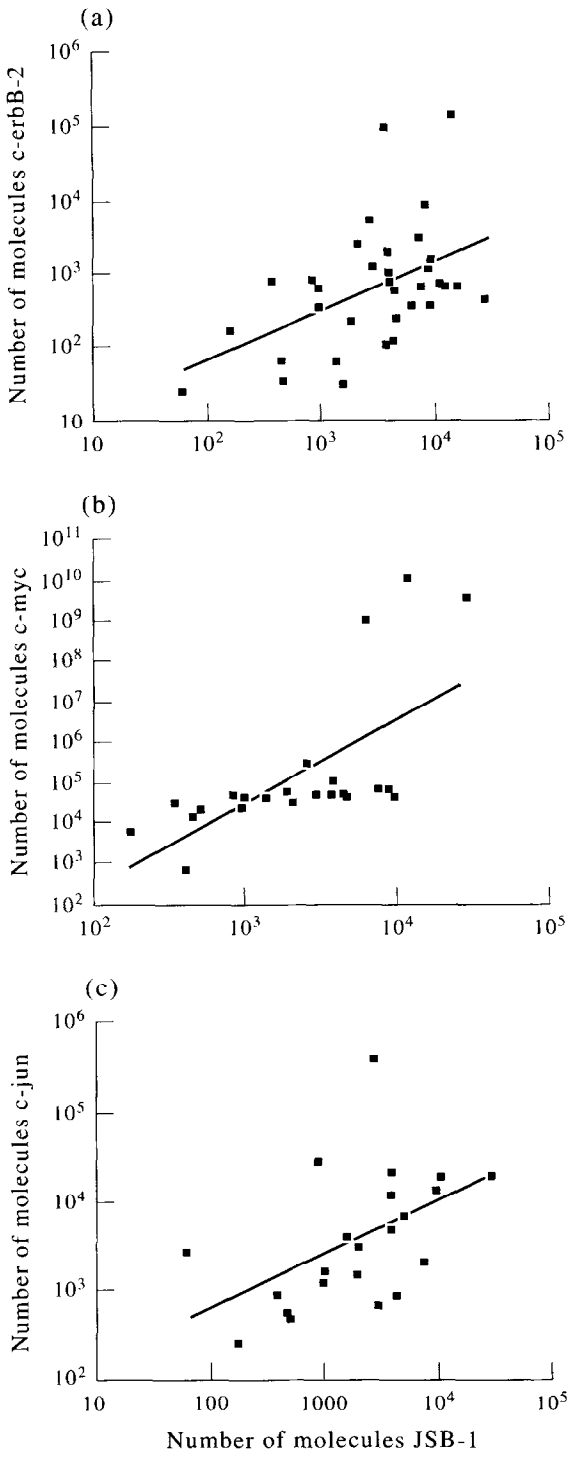

Figure 5. The relationships between JSB-1 and (a) c-erbB-2, (b) c-myc and (c) c-jun. JSB-1 showed a positive correlation with all three markers ($P = 0.003$, $n = 35$, $P = 0.0004$, $n = 25$ and $P = 0.02$, $n = 22$, respectively).

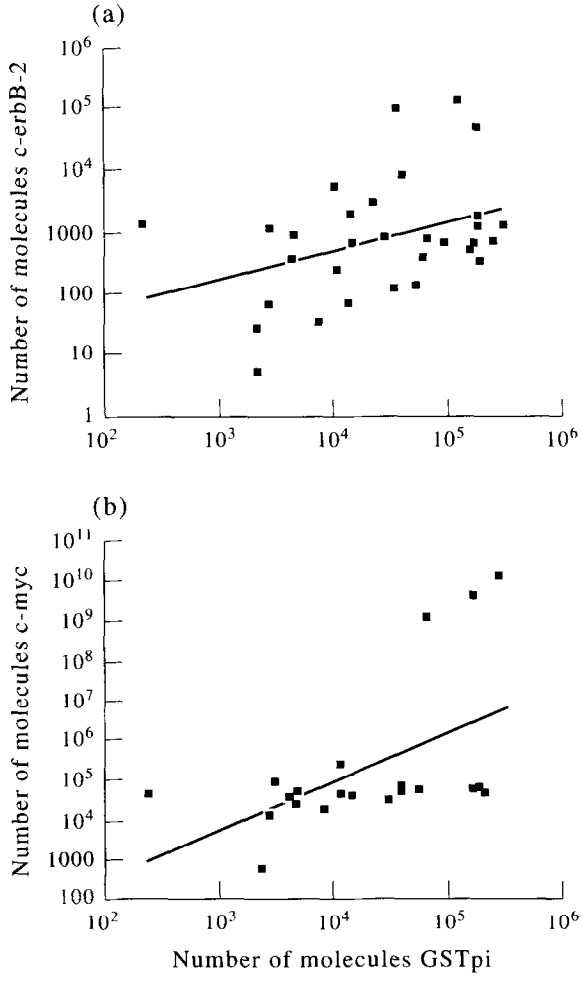

Figure 6. The relationships between GSTpi and (a) c-erbB-2 and (b) c-myc. GSTpi showed a positive correlation with both markers ($P = 0.03$, $n = 35$ and $P = 0.0004$, $n = 25$, respectively).

enhancement of poorly expressed antigens where non-cytokeratin positive cells may obscure the result. In solid tumour samples which are selected histopathologically to contain mainly malignant cells, the need for gating strategies is markedly reduced. In this study, we were limited to a comparison of total MDR expression with cytokeratin gated populations of cells. However, histopathology was used to select for high tumour cell purity, and MDR expression is likely to be similar to that observed here should gating have been possible. The main reason behind gating was to enhance certain types of the more poorly expressed antigens, most notably ER.

Without doubt, the main area of contention concerning the measurement of MDR has been the variable results published thus far [16, 18]. With respect to the use of monoclonal antibodies, the main criticisms levelled at such methods concern the crossreactivity of the antibodies with other proteins [24, 25]. Use of the antibody JSB-1 has been reported to give very good staining results by immunohistochemistry [11], with little non-specific staining, a finding this study supports. Using a panel of known positive and negative cell lines and tissues, we confirmed the expected staining pattern for JSB-1 (Figure 1a). Notably, the the level of expression by liver, a positive control, was high, with the level in the known drug resistant cell line markedly higher.

GSTpi results were not as one would predict. The sensitive cell line MDS (Figure 1b) expressed inexplicably high levels. This finding may account for some of the cell properties in MDR experiments. However, more significantly, the

antibody failed to give a reproducible staining pattern of sufficient intensity with the positive liver control. Despite repeated attempts and altering liver sampling techniques (excluding certain transplant tissues which showed decreased expression of MDR with all antibodies), GSTpi failed to produce satisfactory results. These findings are reflected in the lack of correlation between the two antibodies when measured on cell lines. Notably, levels of GSTpi were higher than JSB-1, which may suggest non-specific binding. Alternatively, it may be a reflection of the inability to quantify numbers of molecules due to methodological constraints, or tissue culture conditions may affect the GSTpi epitope. Furthermore, our data suggest that previously reported non-specific staining patterns may indeed occur and could account for some of the discrepancies in this paper.

JSB-1 expression in primary breast tumours (Figure 2) was higher than those seen on the most positive cell lines, with over half the tumours showing levels of JSB-1 greater than that of the MDR positive cell line. Interestingly, the majority of breast cancer cases assessed expressed some level of MDR using this antibody. Possible explanations include increased sensitivity of MDR detection by flow cytometry or expression of MDR by non-epithelial cells or non-specific labelling. Non-specific binding is not supported by our cell line results. Sufficient expression of MDR by non-epithelial cells cannot be ruled out, although previous work suggests the numbers of non-epithelial cells present are unlikely to be able to alter the result significantly [23]. Employment of backgating techniques to define a FSC versus SSC tumour gate has also been employed, removing non-epithelial cells possessing detectable differences in cell size and granularity.

Levels of GSTpi appeared lower than those expressed by the majority of cell lines examined. This was rather surprising as normal breast ducts and lobules usually show intense staining patterns with GSTpi and, therefore, a high level of non-specific staining would be expected. Whether this is a reflection of the tissue used is unknown, but it confirms our belief that the GSTpi antibody is unsatisfactory for use in flow cytometry. The general staining pattern produced by GSTpi suggests an interaction with some epitope other than the designated target. Whether the relationship between JSB-1 and GSTpi reflects an underlying mechanism involved in MDR is not known. However, a strong correlation existed between these two MDR markers with increased JSB-1 levels associated with increased GSTpi levels.

The question which needs to be addressed is why there is an alteration in the staining ability of tumours compared with cell lines and whether this is related to the tumour cells, stromal cells or indeed non-malignant epithelial cells. More importantly, we need to know what causes this discrepancy and whether it is important in the MDR mechanism.

We did not note any relationship between either marker with tumour grade or axillary node status. This is not too perturbing as they are markers of prognosis and likely to be independent of clinical drug resistance. Of more interest would be the chemotherapeutic regime, relapse and survival data for these patients, none of which were available at this time.

Data showing the relationship of other markers with JSB-1 and GSTpi using cytokeratin gated populations has revealed a positive association between JSB-1 and c-erbB-2, c-myc and c-jun, while GSTpi correlated with c-erbB-2 and c-myc. Notably, the markers of prognosis ER and EGF-R showed no correlation with MDR.

The proto-oncogene *c-myc* produces a nuclear protein that may have a role in DNA synthesis [26]. Amplification of the *c-myc* gene has been found in several types of human tumours including breast [27]. Overexpression of *c-myc* is known to be an important step in tumour progression, and it is frequently expressed in proliferating and transformed states, being associated with breast tumour grade [27, 28]. The link between a high *c-myc* expression and cancer has been demonstrated by construction of transgenic mice carrying a *c-myc* gene under control of a hormonally inducible mouse mammary tumour virus promoter [29]. These mice spontaneously developed mammary adenocarcinomas.

With the correlation between MDR and c-myc, these markers may indicate progression and proliferation within the tumour, and indicate that chemotherapeutic regimes may select the MDR/c-myc positive cell, due to the death of cells expressing the MDR negative phenotype.

Expression of *c-erbB-2,* with its known homology with EGF-R, has been associated with faster tumour proliferation and poorer prognosis [30, 31]. This increased tumour proliferation would fit in with the increased levels of c-myc. Overexpression of *c-erbB-2* is also a marker of poor response to both endocrine and conventional cytotoxic therapy [32–34]. Whether this finding is due to the development of multidrug resistance or caused by it is not yet known.

We conclude indirectly that examination of *MDR* expression may indicate the possibility of a poorer prognosis. Clinical data for chemotherapeutic treatments, survival and relapse time are not available at this time. However, if negative correlations can be drawn between *MDR* expression and relapse-free and overall survival, then measurement of MDR by flow cytometry may be an important method for monitoring tumour progression. However, the discrepancies in expected staining patterns, most notably using the GSTpi antibody, and to a lesser extent the high level of *JSB-1* expression exhibited by breast tumours, indicate a cautionary approach to the measurement of MDR. Further study is needed to examine the reasons for these occurrences prior to any clinical use.

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