

MONOCLONAL ANTIBODY JSB-1 DETECTS A HIGHLY CONSERVED EPITOPE ON THE P-GLYCOPROTEIN ASSOCIATED WITH MULTI-DRUG-RESISTANCE

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Resistance to multiple chemotherapeutic agents is a common clinical problem in the treatment of cancer. This resistance may occur before primary therapy or be acquired during treatment. We have generated a monoclonal antibody (MAb) (JSB-1), specific for a conserved epitope on the plasma membrane 170- to 180-kDa glycoprotein, the expression of which is strongly correlated with the degree of multi-drug resistance (MDR). JSB-1 strongly binds to both Chinese-hamster-derived MDR cell lines and human MDR cell lines, including cell lines derived from lung and ovary. A drug-sensitive revertant line, and the corresponding drug-sensitive parent lines, showed only weak reactivity or none at all. JSB-1 reacts strongly to air-dried or acetone-fixed cells and therefore has potential value for diagnostic detection of MDR cells in human tumor samples.

Development of drug resistance in tumors during chemotherapy limits the use of cytostatic drugs. Clinically, tumors resistant to one drug are often cross-resistant to other drugs of various classes. During the past 10 years, a new system has been defined which partly explains drug resistance of cancer cells. This system is termed multi-drug resistance (MDR; Biedler and Riehm, 1970; Bech-Hansen *et al.*, 1976; Skovsgaard, 1978; Beck, 1984). This type of resistance is often due to the amplification of one gene, the MDR-1 gene, that is transcribed into a 4.5-kb mRNA (Gros *et al.*, 1986a,c; Roninson *et al.*, 1986; Jongsma *et al.*, 1987). The protein product of this gene is a high-molecular-weight membrane glycoprotein, termed P-glycoprotein or gp 170 (Juliano and Ling, 1976; Kartner *et al.*, 1983). This membrane protein controls the efflux of drugs from the cell. Cells having a large amount of this protein are assumed to expel the drug at a faster rate (Gerlach *et al.*, 1986; Chen *et al.*, 1986).

Despite the importance of the MDR system for resolving several fundamental questions related to cell handling of harmful agents, as well as for clinical and diagnostic purposes, only a few MAbs to human P-glycoprotein have so far become available. Some of these react with epitopes exposed on viable cells (Danks *et al.*, 1985) and may functionally affect drug transport (Hamada and Tsuruo, 1986), whereas other MAbs are directed against a very well-conserved cytoplasmic region of P-glycoprotein (Kartner *et al.*, 1985). By immunoblotting techniques, one of these MAbs (C219) has been successfully used to detect over-expression of P-glycoprotein in tumor samples from advanced ovarian cancer (Bell *et al.*, 1985) and sarcoma patients (Gerlach *et al.*, 1987). The MDR phenotype has been detected in 2 patients with drug-resistant acute non-lymphoblastic leukemia by means of an immunocytochemical assay with the C219 MAb (Ma *et al.*, 1987). These findings prompted us to attempt to raise new P-glycoprotein-specific MAbs that might contribute to a better understanding of the MDR phenomenon.

MATERIAL AND METHODS

Tumor cell lines

Two MDR Chinese hamster cell lines, as well as the parent cell lines, were used (Table I). The adenosine-, thymidine-

and glycine-requiring auxotroph AuxB1 of Chinese hamster ovary cells, and its colchicine-selected mutant CHrC5 (Ling and Thompson, 1974) were obtained from Dr. V. Ling of the Ontario Cancer Institute, Toronto, Canada. The Chinese hamster lung cell line DC3F, and its daunorubicin-selected mutant DC3F/DMXX (Biedler *et al.*, 1983), were obtained from Dr. J.L. Biedler, Memorial Sloan-Kettering Institute, New York.

Three human multi-drug-resistant carcinoma cell lines, as well as the drug-sensitive parent cell lines, were studied. The multi-drug-resistance phenotype in all 3 lines had been induced by stepwise selection with doxorubicin (adriamycin): in ovary cells by one of us (H134AD, Broxterman *et al.*, 1987b) and, independently, by Rogan *et al.* (2780AD, 1984), and in human lung squamous-cell carcinoma-derived cells obtained from Dr. H.G. Keizer (Department of Anthropogenetics, Free University, Amsterdam; SW1573AD).

Multi-drug-resistant cell lines were maintained in the continuous presence of colchicine (5 µg/ml, CHrC5) daunorubicin (10 µg/ml; DC3F/DMXX) or doxorubicin (H134AD, 3 µM, 2780AD, 2 µM, SW1573AD, 0.5 µM) respectively. A revertant cell line, showing residual 8-fold resistance, was obtained from the 2780AD cell line (160-fold resistant) by omitting doxorubicin from the culture medium for several months of culture (2780Rev; Broxterman *et al.*, 1987a).

Immunization and cell fusion

Female 8-week-old BALB/c mice received 2 injections of 1×10^7 CHrC5 cells in complete Freund's adjuvant (CFA; Difco) i.p. and s.c. into the nape of the neck 3 days after a supposedly immunopotentiating dose of cyclophosphamide (Endoxan, ASTA, Brackwede, FRG; 20 mg/kg, Livingston *et al.*, 1985). This was followed by 4 weekly booster injections of 1×10^6 CHrC5 cells: 3 times i.p., once i.v.

Fusion of the mouse spleen cells and SP2/O myeloma cells was performed 4 days after the last booster injection, and hybridomas were selected in hypoxanthine/aminopterin/thymidine medium as described by Lane (1985). Hybrid cells secreting antibodies of interest were cloned by limiting dilution. To obtain a large quantity of antibody, hybrid cells were injected into the peritoneal cavity of BALB/c mice receiving pristane (Aldrich, Milwaukee, WI) and ascites was collected subsequently.

MAb screening

Initial screening of hybridoma cultures was performed on cell smears of CHrC5 and the drug-sensitive parent cell line. In further studies, cytocentrifuge preparations were used. To select for MAbs detecting epitopes shared with human multi-

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drug-resistant tumor cells, the human ovary cell line H134AD was used for secondary screening. An attempt to use cells of this line for both immunization and primary screening was unsuccessful.

Cell smears and cytospin preparations were air-dried, fixed in acetone for 10 min and incubated for 60 min with culture

supernatants. After washing with 0.01 M phosphate-buffered saline (PBS), slides were covered with a 1:100 dilution of rabbit anti-mouse Ig peroxidase (DAKO, Copenhagen, Denmark). Slides were subsequently stained for peroxidase activity with 3,3'-diamino-benzidine-tetrahydrochloride (DAB, Sigma, St. Louis, MO):0.5 mg/ml PBS containing 0.015% H_2O_2 . Control slides were incubated in the same way, omitting the first step, or using unrelated supernatants.

Percentages of cells stained with the antibody JSB-1 were determined by counting at least 200 cells per preparation, whereas positive cells were classified as either weakly, intermediately or strongly staining.

Isotype determination

The isotype of the MAb of interest, designated JSB-1, was determined in an indirect 2-step ELISA method. Briefly, plates coated with rabbit anti-mouse isotype-specific antibodies (Miles, Elkhart, IN) were incubated with appropriate supernatant, after which binding was detected with a peroxidase-conjugated rabbit anti-mouse antiserum as described above.

Immunoprecipitation and electrophoresis

CHrC5 and AuxB1 cells were both surface labelled with ^{125}I by Iodogen. The cells were lysed at 4°C for 30 min in a

TABLE I - DESCRIPTION OF TUMOR CELL LINES

Parental line	Resistant line	Drug of selection	RR ¹
Chinese hamster ovary AuxB1	CHrC5	Colchicin	200×
Human ovary A2780	2780AD	Doxorubicin	160×
H134	H134AD	Doxorubicin	250×
Chinese hamster lung DC3F	DC3F/ DMXX	Daunorubicin	880×
Human lung SW1573	SW1573AD	Doxorubicin	150×

¹Relative resistance to cytotoxic drug used for selection. RR is calculated by dividing the concentration of drug required to inhibit growth at cells by 50% (IC_{50}) in 48 hr by the IC_{50} in the drug-sensitive parent cell line.

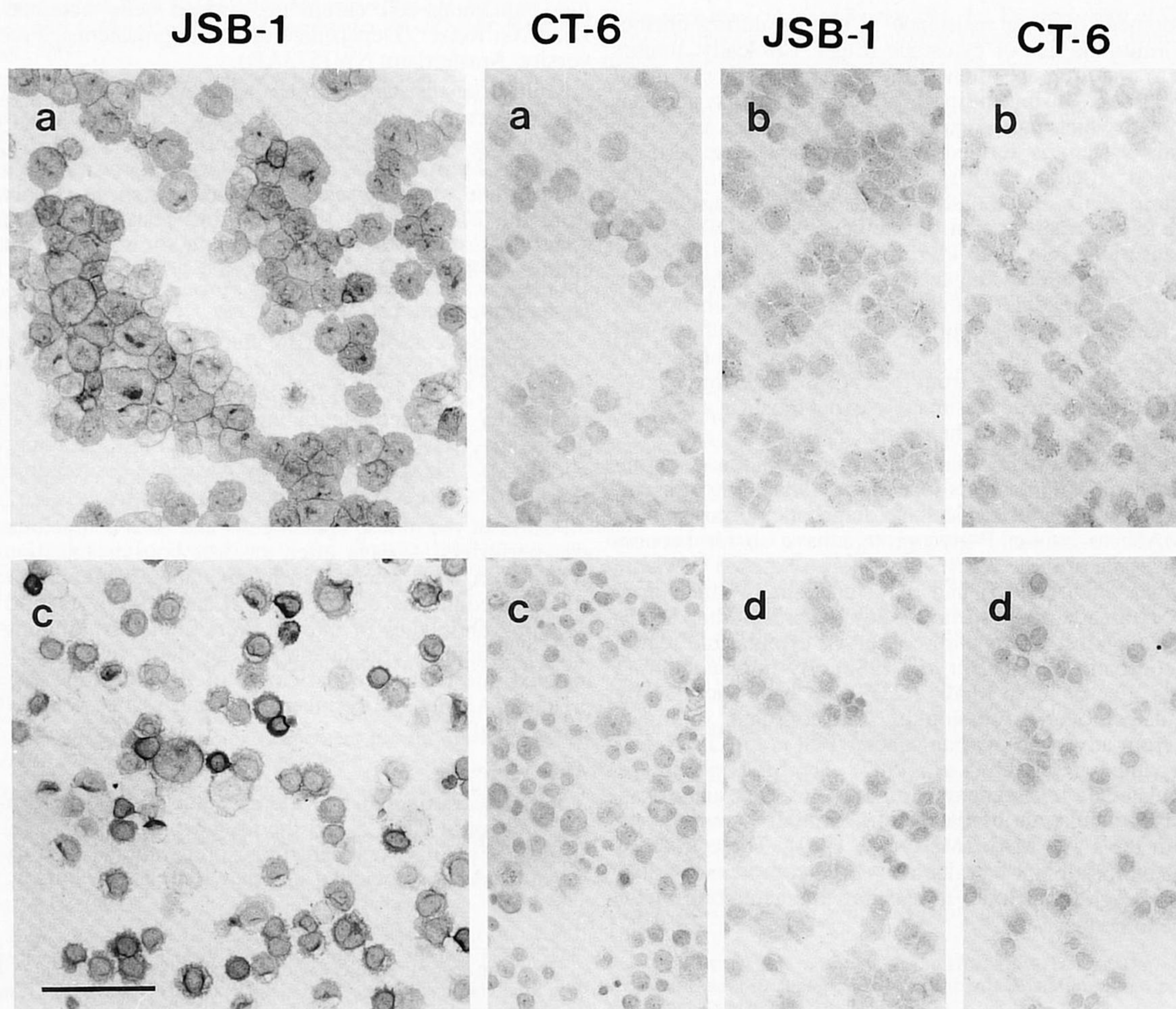


FIGURE 1 - Peroxidase-staining of cytocentrifuge preparations of drug-resistant Chinese hamster CHrC5 (a) and human H134AD (c) cell lines with the JSB-1 antibody. Drug-resistant cells treated with a control antibody (CT-6) and drug-sensitive parent cell lines AuxB1 (b) and H134 (d) show background methylene blue counterstaining (bar = 50 μ m).

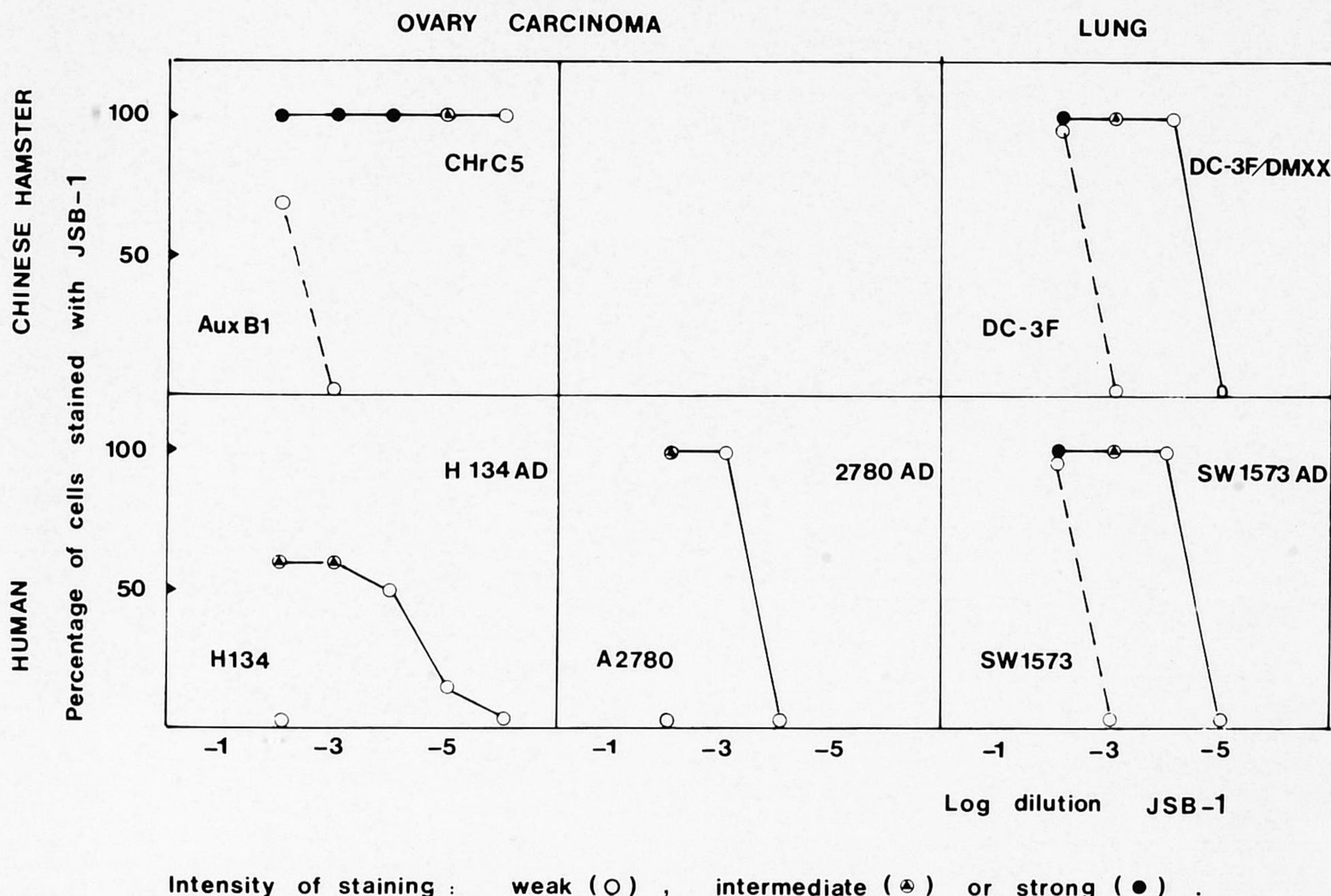


FIGURE 2 - JSB-1 staining of 2 couples of Chinese hamster (CHrC5 and AuxB1, DC-3F and DMXX-DC-3F) and 3 couples of human (H134AD and H134; 2780AD and A2780; SW1573AD and SW1573) ovary and lung cell lines. Both percentages of cells stained and average staining intensities are given for serial dilutions of JSB-1 ascites.

0.01 M Tris-HCl buffer, pH 7.8, containing 1% (v/v) NP40, 0.15 M NaCl, 1mM PMSF, 5mM Na₂ EDTA and 0.02 mg/ml trypsin inhibitor. NP40 lysates, pre-cleared with pre-formed complexes of normal mouse serum/goat anti-mouse (GAM) serum, were incubated with MAb-GAM complexes for 16 hr at 4°C. The precipitates were centrifuged on a discontinuous sucrose gradient and boiled for 5 min in 50 μ l sample buffer containing 6% SDS and 10% B-mercaptoethanol. Precipitates were electrophoresed on SDS gels of 7% polyacrylamide (w/v) according to Laemmli (1970). For autoradiography, Kodak X-Omat RP X-ray film was used.

RESULTS

Generation of MAbs

Of the 282 primary culture supernatants assayed, 199 reacted to both CHrC5 and AuxB1 cell lines. Two bound specifically to CHrC5, and one of these, designated JSB-1, was highly cross-reactive with the human multi-drug-resistant ovary cell line H134AD (Fig. 1). Hybridoma cells producing the antibody were repeatedly subcloned and proved to be stable over several weeks of *in vitro* culture. The cell line is consistently recovered from the frozen state. In pristane-primed BALB/c mice, the cell line produces an ascitic tumor secreting antibody JSB-1. The antibody belongs to the immunoglobulin subclass IgG₁.

Specificity of JSB-1 tested with a panel of MDR cell lines

To verify the binding specificity of JSB-1 for a conserved epitope associated with MDR cells, a larger panel of drug-

sensitive and multi-drug-resistant cell lines (Table I) was screened. Drug-resistant Chinese hamster cells derived from both ovary and lung cell lines strongly bound JSB-1 (Fig. 2). All 3 human ovary- and lung-derived MDR cell lines tested were also strongly stained by JSB-1, confirming the well-conserved nature and prevalence of the epitope recognized by this MAb. Weak but definite staining could be detected in some drug-sensitive parent cell lines of both Chinese hamster (AuxB1 and DC3F) and human (SW1573) origin. Cell staining was due to binding to a cytoplasmic component closely associated with the plasma membrane. Distinct intracytoplasmic staining regions, suggesting Golgi involvement, were often observed (Fig. 3).

To further confirm the association of JSB-1 positivity with expression of the MDR phenotype, the human drug-sensitive revertant cell line 2780Rev was included in the panel. Even at the highest dilution of JSB-1 tested (1:10 diluted ascites) no positive staining of these cells could be detected.

Binding properties of JSB-1

Some degree of permeabilization of MDR cells was a prerequisite for exposing the epitope recognized by JSB-1: no staining of viable, intact cells could be detected. Air drying of cytospin preparations was sufficient to allow access to the epitope, and acetone fixation neither promoted nor impaired cellular staining (Table II). Methanol and formaldehyde/ethanol/acetone fixation, however, rendered the epitope less reactive to binding.

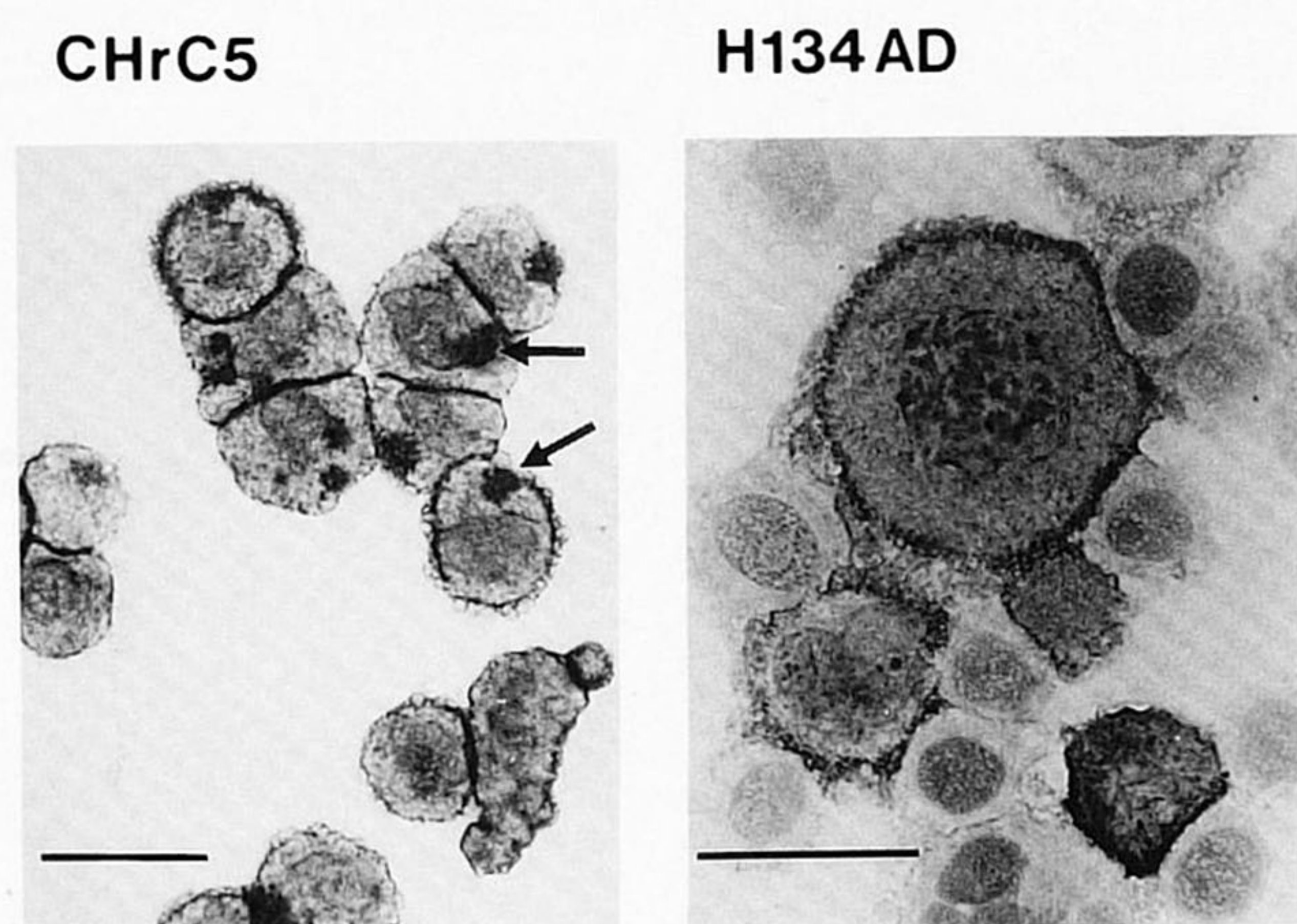


FIGURE 3 - JSB-1-stained cyto centrifuge preparations of CHrC5 and H134AD cell lines showing specific binding to a cytoplasmic component closely associated with the plasma membrane and to distinct intracytoplasmic regions (arrows; bar = 25 μ m).

Identity of the antigen recognized by JSB-1

To confirm the reactivity of JSB-1 with P-glycoprotein, we performed immunoprecipitation experiments with JSB-1 on surface-labelled CHrC5 cells. As a control, the drug-sensitive AuxB1 parent cell line was used. Indeed, JSB-1 precipitated a 170 kDa band from CHrC5 cells. This band was not seen either in the immunoprecipitate from AuxB1, or when an irrelevant MAb of the IgG₁ isotype was used instead of JSB-1 (Fig. 4).

DISCUSSION

To study the problem of multi-drug resistance, several laboratories, including ours, have isolated cell lines resistant to the Vinca alkaloids, doxorubicin, actinomycin D, colchicin and related agents. A consistent finding of *in vitro* studies of MDR cell lines has been the over-expression of P-glycoprotein (gp-170) in these cells. Initially described in MDR Chinese hamster cell lines by Juliano and Ling (1976), the association between P-glycoprotein over-expression and drug resistance has been confirmed by a large number of subsequent studies (Pastan and Gottesman, 1987). Remarkably, there are still very few MAbs which react specifically with this high-molecular-weight glycoprotein. The weak but distinct expression of the MDR-1 gene in several normal tissues (Fojo *et al.*, 1987), as well as the strong homology between the MDR-1 gene product and functionally related proteins in mammals, suggest low antigenicity of the molecule. Moreover, the strongly hydrophobic character of P-glycoprotein, related to 12 separate transmembrane regions (Gros *et al.*, 1986b), might add to poor access of potentially antigenic epitopes to immune effector cells.

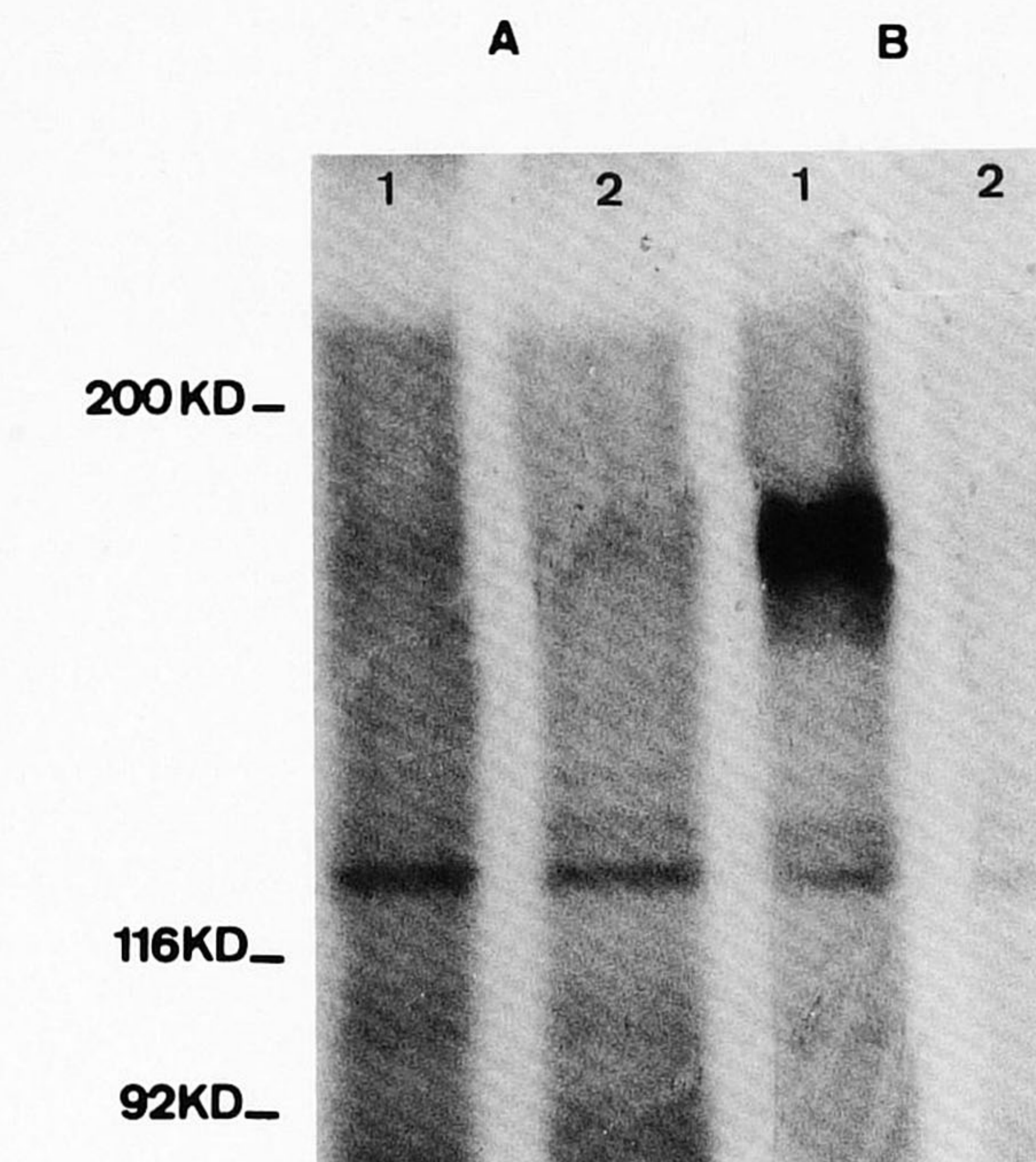


FIGURE 4 - Radioimmunoprecipitation of ¹²⁵I-surface-labelled AuxB1 (a) and CHrC5 (b) cells with JSB-1 (lanes 1) or a control antibody (lanes 2).

This view is supported by our present findings. So far, all attempts to produce P-glycoprotein-specific MAbs by immunizing mice with the human MDR ovary cell line have failed. Using the CHrC5 cell line, we were able to obtain one hybridoma producing a putative gp170-specific MAb (JSB-1). As a corollary to our method of selection, this MAb shows excellent immunostaining of cytological preparations. JSB-1 strongly cross-reacts with the lung-derived Chinese hamster MDR line DC3F/DMXX and, more importantly, also shows strong binding to all human MDR lines tested so far, including cell lines derived from lung and ovary. In addition, JSB-1 reacted strongly with human RPMI 8226-derived MDR myeloma cell lines (Dalton *et al.*, 1986), showing binding that closely paralleled the degree of drug resistance (data not shown).

Based on the correlation of increased expression of the antigen with increased MDR, and the conservation of the antigen irrespective of the drug of selection in different mammalian species, we conclude that the antigen detected is P-glycoprotein. Immunoprecipitation with JSB-1 of a 170-kDa protein from the CHrC5 cell line confirms this conclusion. Two preliminary attempts to precipitate a similar 170-kDa molecule from the human H134AD cell line failed, suggesting lower affinity of JSB-1 for the human epitope. Interestingly, similar difficulties were met with the human P-glycoprotein-specific MAbs MRK 16 and 17 (Hamada and Tsuruo, 1987).

TABLE II - EFFECTS OF FIXATION ON STAINING WITH JSB-1

Fixation of cyto centrifuge preparations	Chinese hamster		Human MDR lines		
	CHrC5	DC3F/DMXX	H134AD	2780AD	SW1573AD
Air-dried, unfixed	+++ ¹	+++	++	++	+++
Acetone, 10 min 20°C	+++	+++	++	++	+++
Methanol, 5 min -20°C	+++	+	++	+	+
Formaldehyde, 0.5%, 5 min 0°C; Ethanol, 95%, 5 min -20°C; Acetone, 5 min -20°C	+	++	+	-	+

¹Intensity of staining: strong (+++), intermediate (++), weak (+) or negative (-).

Nevertheless, confirmation that in human MDR cells JSB-1 also detects a 170-kDa molecule has been obtained in Western blots using membrane protein preparations from the human 8226/DOX myeloma MDR cell line (data not shown).

In our hands JSB-1 did not detectably bind to the surface of intact, multi-drug-resistant cells. After some degree of membrane permeabilization, however, strong cytoplasmic staining, preferentially at the cytoplasmic aspect of the plasma membrane, could be detected. A region located in the C-terminal fragment, and containing the epitope recognized by C219, has been thought to be more immunogenic than most of the protein (Kartner *et al.*, 1985). It is therefore tempting to conclude that JSB-1 reacts to an epitope closely linked to the one recognized by C219. Differences between JSB-1 and C219 include their isotypes (IgG₁ and IgG₂ respectively), as well as the good performance of JSB-1 in immunocytochemical staining. Further identification of the epitopes recognized by both MAbs will obviously need cross-binding studies.

Although staining with most MDR cell lines tested was remarkably homogeneous, some degree of heterogeneity could be observed, in particular with the H134AD cell line (Figs. 2, 3). At this stage it is not clear how far a variable degree of epitope expression is cell-cycle-related, or due to uneven epitope-inactivation during processing. Alternatively, MDR in a cell line may reflect, in addition to P-glycoprotein-related MDR, other mechanisms acting in concert. This hypothesis is currently studied at the clonal level for the H134AD cell line. The remarkable intra-cytoplasmic, probably Golgi-related, staining observed in most MDR cell lines studied suggests that JSB-1 recognizes an epitope generated early during protein maturation.

As might be expected from the presence of MDR-1 gene-

coded mRNA in various normal tissues and cell lines, not selected for drug resistance (Fojo *et al.*, 1987), faint staining could be detected with JSB-1 in some parent cell lines (AuxB1, DC3F and SW 1573). Interestingly, absence of even the faintest staining was noted in the 2780AD-revertant cell line, despite a distinct residual degree of drug-resistance in these cells. This may be simply due to the inability of JSB-1 to detect low levels of P-glycoprotein in this particular cell line, since no staining of the parent line cell line (A2780) was observed. Alternatively, it may also indicate that mechanisms other than over-expression of the gp 170 molecule may contribute to MDR (Beck, 1984).

The emergence of MDR cancer cells during the course of chemotherapy continues to be a major problem. It is recognized with great frequency in patients treated for ovarian cancer, breast cancer, lymphoma and leukemia. Screening of multiple myeloma specimens has shown positive staining with JSB-1 in several patients (not presented). For reasons as yet unknown, over-expression of P-glycoprotein may be particularly prevalent in human sarcomas (Gerlach *et al.*, 1987). Determination of the ultimate clinical significance of P-glycoprotein staining will need controlled studies which relate levels of P-glycoprotein expression to disease outcome with defined chemotherapy in prospective clinical protocols. The MAb JSB-1 provides a sensitive and specific reagent with which to perform such studies.

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