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Detection of the Multidrug Resistant Phenotype in Human Tumours by Monoclonal Antibodies and the Streptavidin–Biotinylated Phycoerythrin Complex Method

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Abstract—*The aim of this study was to find out whether the membrane glycoprotein P-170 can be detected in human tumours with both acquired and intrinsic resistance to chemotherapeutic agents using monoclonal antibodies (265/F4 and C219) and the streptavidin–biotinylated phycoerythrin complex method. Pretreated leukaemia cells and untreated lung and ovarian carcinomas were analysed. Two plasmacytomas and one leukaemia expressed high levels of P-glycoprotein, whereas two leukaemias showed moderate, and three leukaemias no expression of this protein. The intrinsic resistance was analysed with a panel of four human epidermoid lung cancer xenografts grown in nude mice. The expression of P-glycoprotein could be correlated with the degree of resistance. In addition, one out of five ovarian carcinomas revealed a high level of P-glycoprotein.*

INTRODUCTION

STUDIES performed with cultured tumour cell lines selected for resistance to a single drug have shown that cross-resistance between anthracyclines, dactinomycin and vinca alkaloids is a common phenomenon [1, 2]. This phenomenon has been designated as multidrug resistance or pleiotropic resistance. The most frequently reported alteration of multidrug-resistant cells is the overexpression of a 170 kD membrane glycoprotein (P-170 or P-glycoprotein) originally described by Ling and coworkers [3].

While this drug-resistant phenomenon has been most extensively studied in animal and human cell systems in tissue culture, the data on tumours grown *in vivo* is sparse. Preliminary reports have indicated that P-glycoprotein is also overexpressed in human drug-resistant solid tumours and human leukaemia cells. Bell *et al.* [4] could detect the plasma membrane protein P-170 in two out of five pretreated patients with resistant ovarian carcinomas using Western blot analysis. Ma *et al.* [5] detected a multidrug-resistance phenotype in two patients with drug-resistant leukaemias by an immunocytochemical assay using a monoclonal antibody to P-glycoprotein. Gerlach *et al.* [6] detected the P-glycoprotein by Western blot analysis in six out of 25 sarcomas, while 35 other tumours of different types were negative. In addition, Tsuruo *et al.* [7] found that three out of six patients with chronic myelogenous leukemia blast crisis expressed high levels of mRNA which codes for P-glycoprotein. In spite of these data, up to now it is unclear whether the overexpression of P-glycoprotein is responsible for the failure of chemotherapeutic regimens [8].

The aim of this investigation was to find out whether P-glycoprotein is increased both in intrinsic and acquired resistance of human tumours towards chemotherapeutic agents using monoclonal antibodies and the recently developed very sensitive streptavidin–biotin–phycoerythrin method. Therefore, we analysed pretreated leukaemia cells and untreated lung and ovarian carcinomas.

MATERIALS AND METHODS

Animal tumours

The development of resistant murine leukaemia L1210 and murine sarcoma 180 (S180) ascites tumour lines has been described previously [9, 10]. The degrees of resistance of doxorubicin-resistant

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L1210 cells [$L1210_{DOX}$] to doxorubicin is 45-fold, to daunorubicin 24-fold and to dactinomycin 12-fold, whereas a cross-resistance to cytosine-arabinoside does not exist. The degrees of resistance of daunorubicin-resistant S180 ascites cells [$S180_{DNR}$] are 73-fold to daunorubicin, 275-fold to doxorubicin and 50-fold to dactinomycin. This cell line showed a collateral sensitivity to cytosine-arabinoside. Thus, we have a multidrug-resistance phenotype in both investigated tumour cell lines grown *in vivo*.

Human xenografts. In order to establish human lung tumour lines with different and detectable resistance to drugs, samples of human epidermoid lung carcinomas obtained from the Rohrbach-Heidelberg Chest Hospital were transplanted into nude mice. All tumours had not received prior chemo- or radiotherapy in the clinical stage or as xenografts in nude mice. The tumour lines were maintained by serial subcutaneous transplantation of minced tumours into the right subaxillary region. After the tumour reached a diameter of 8–10 mm, the tumour-bearing mice were randomized into groups of 5–7 animals each, treatment (i.p.) with a single dose of 2 mg/kg BW vincristine and 0.5 mg/kg BW dactinomycin, respectively, was started and the therapeutic effect was determined. This model was used because xenograft lines derived from human tumours revealed different degrees of resistance to vincristine or dactinomycin (Table 1). The collateral sensitivity to alkylating agents and to antimetabolites, which is often observed in a number of multidrug-resistant cell lines [11], is also found in the resistant human tumour lines as described earlier [12].

Human tumours. Surgical specimens of untreated ovarian carcinomas of stage III and IV patients were acquired at the Department of Obstetrics and Gynaecology of the University of Freiburg. All specimens were freed from necrotic and normal tissue parts, then kept frozen at −80°C until the time of processing. Leukaemia cells and bone marrow aspirates were collected at the Polyclinic of the University of Heidelberg. Only fresh tumour material was processed. The leukaemia cells were separated by Ficoll–Hypaque density gradient centrifugation. All patients had been pretreated with different chemotherapeutic agents usually of the multidrug pattern (anthracyclines, alkaloids).

Table 1. Response of different human xenografts of the lung (HXL) to the cytostatics vincristine and actinomycin D. Relative tumour size on day 3 after application of the cytostatics

Xenografts		Response (% of control) Vincristine	Actinomycin D
HXL 54	Resistant	88	93
HXL 204		74	77
HXL 182		56	69
HXL 55	Sensitive	21	48

Immunofluorescence. For immunofluorescence detection of P-glycoprotein we used the streptavidin–biotin–phycoerythrin method recently developed by Amersham. Tumour cells were suspended in Hank's salt solution and centrifuged by Cytospin 2 (Shandon) resulting in a cell monolayer on the slides. Cryostat sections were performed on solid tumours. After air drying, fixation of cells and sections in acetone was carried out as a permeabilization step. After incubation of cells and cryostat sections with normal sheep serum the primary monoclonal antibodies (265/F4 or C219) were applied (10 μg/ml) for 2 h. After washing, the cells were incubated with biotinylated sheep antimouse second antibody (dilution 1:50, 30 min, Amersham, pooled with 5% human IgG) and after rewashing in PBS, the streptavidin–biotinylated phycoerythrin complex method (Amersham) was carried out (dilution 1:50, 40 min). After addition of a stabilizer for 20 min to prevent rapid fading of phycoerythrin fluorescence, the slides were dried and mounted.

The preparation and characterization of the monoclonal antibodies have been described earlier [13, 14]. The antibodies were kind gifts from Dr. B. Lathan, Cologne, FRG and Dr. V. Ling, Toronto, Canada. These antibodies were prepared against the membrane P-glycoprotein (M_r 170 kD) in colchicine-resistant Chinese hamster ovary (CHO) cells.

Immunoblotting

The isolation of plasma membranes was performed according to Riordan and Ling [15]. SDS-PAGE was carried out in a slab gel apparatus according to Fairbanks *et al.* [16]. Protein concentration in the different protein extracts was determined to the method of Bradford [17] and immunoblotting to Towbin *et al.* [18] and Lathan *et al.* [13].

RESULTS

Animal tumours

In order to evaluate whether resistant tumour cells grown in animals show alterations in the content of P-glycoprotein of the plasma membranes, the streptavidin–biotin–phycoerythrin method and two antibodies against P-glycoprotein were used. Figure 1 IA demonstrates that resistant L1210 ascites tumour cells show an intense immunofluorescence reaction whereas no specific immunoreaction was observed in parental (sensitive) cells (Fig. 1

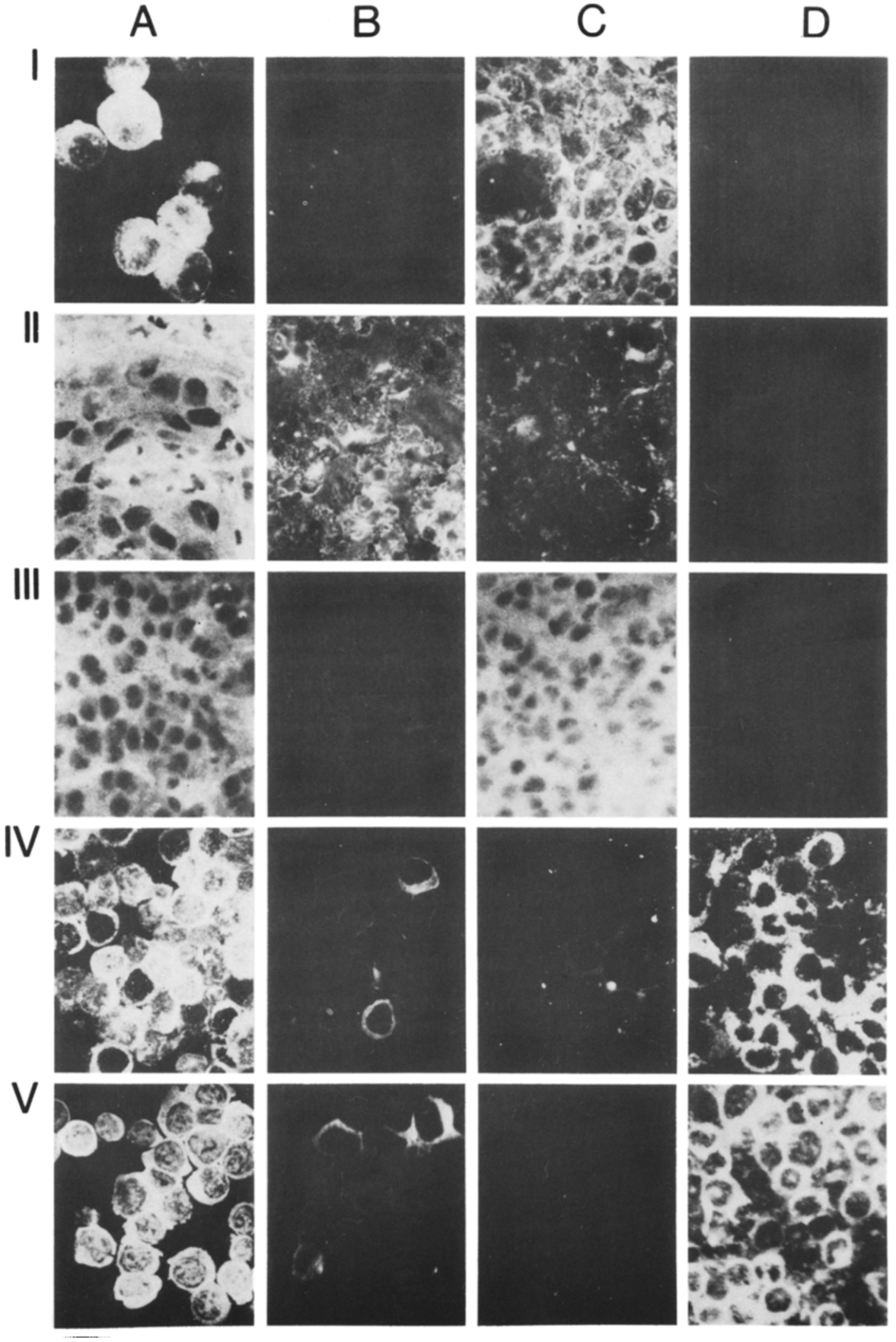

Fig. 1. *Detection of P-glycoprotein in different tumours using the streptavidin–biotin–phycoerythrin immunofluorescence method. Line I: A: doxorubicin-resistant L*1210 *murine ascites tumour cells, B: sensitive (parental) L*1210 *ascites tumour cells (L*1210*), C: daunorubicin-resistant S*180 *solid murine tumour grown in a nude rat, D: sensitive (parental) S*180 *solid tumour (S*180*). Mab* 265/F4. *Line II: Xenografts of human lung carcinoma (HXL) with decreasing resistance to vincristine and dactinomycin (A: HXL*54*, B: HXL*204*, C: HXL*182*, D: HXL*55*). Mab* 265/F4. *Line III: Human ovarian carcinomas. A, C: tumour with overexpression of P-glycoprotein (tumour* 1*), B, D: tumour without overexpression of P-glycoprotein (tumour* 2*). A, B: Mab* 265/F4; *C, D: Mab C*219. *Lines IV and V: Human leukaemia (HL) and plasmocytoma (HP). A–C: human leukaemias with decreasing percentage of fluorescent positive cells (A: HL*1*, B: HL*7*, C: HL*5*), D: plasmocytoma HP*2. *Line IV: Mab* 265/F4; *Line V: Mab C*219. *Magnification:* × 380.

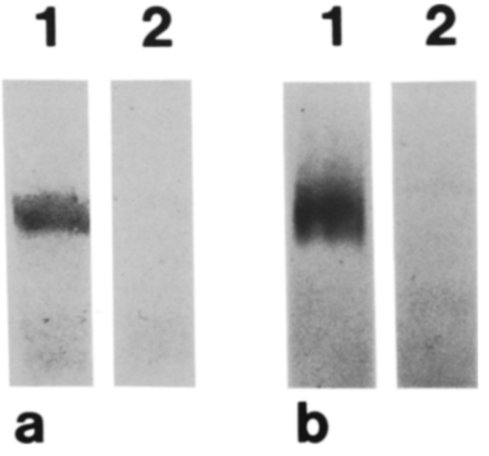

Fig. 2. Immunoblots (Western blots) of plasma membrane extracts (10 μg protein was loaded per lane). (a) Doxorubicin-resistant (1) and sensitive (2) L 1210 ascites tumour cells probed with Mab 265/F4, (b) daunorubicin-resistant (1) and sensitive (2) S180 solid tumours probed with Mab C219 (bands: M_r 170 kD).

IB). As in resistant L1210 tumour cells the P-glycoprotein could be demonstrated in resistant solid tumour specimens generated from S180 ascites tumour cells in nude rats (Fig. 1 IC), but not in specimens of sensitive tumours (Fig. 1 ID). The specificity of the immunostaining could be confirmed by immunoblotting (Fig. 2).

Human xenografts

In order to analyse whether P-glycoprotein can be detected in human tumours not previously treated by chemotherapy, we investigated the intrinsic resistance of a panel of human epidermoid lung cancer xenografts grown in nude mice. Using Mab 265/F4 and the immunofluorescence method we could demonstrate that epidermoid lung carcinomas showed an immunoreactivity according to the degree of resistance (Fig. 1 IIA–IID). Both antibodies showed identical results (data with antibody C219 are not shown).

Human ovarian carcinomas and leukaemias

Solid specimens of not previously treated ovarian carcinomas from five stage III and IV patients were surveyed for P-glycoprotein by immunofluorescence (Fig. 1 IIIA–D). We found a high immunostaining in one ovarian carcinoma (Fig. 1 IIIA,IIIC), whereas in the other specimens no or low immunostaining could be detected (for instance, Fig. 1 IIIB, D).

The expression of P-glycoprotein on pretreated human leukaemia (HL) cells by immunofluorescence is shown in Table 2. Leukaemia HL1 revealed 65 and 67% fluorescence-positive cells, respectively, leukaemia HL6 and HL7 had 1–3% positive cells, leukaemia HL2 less than 1% and leukaemias HL3, HL4 and HL5 were negative. Both plasmacytomas (HP) were positive. In Fig. 1 the human leukaemias HL1, HL7, HL5 (IVA–IVC and VA–VC) and the plasmocytoma HP2 (IVD, VD) are listed. These results demonstrate that P-glycoprotein of human pretreated haematological malignancies and untreated ovarian carcinomas can be detected by the streptavidin–biotinylated phycoerythrin method.

Table 2. Expression of P-glycoprotein of leukaemia cells by indirect immunofluorescence. Quantitative analysis (% of fluorescent positive cells)

	Antibodies	
	265/F4	C219
Controls		
$L1210_{Sens}$	0	0
$L1210_{DOX}$	72	71
$S180_{Sens}$	0	0
$S180_{DNR}$	69	66
Leukaemia		
HL1	65	67
HL2	<1	<1
HL3	0	0
HL4	0	0
HL5	0	0
HL6	2	1
HL7	4	3
Plasmocytoma		
HP1	9	—
HP2	47	52

DISCUSSION

Many laboratories have attempted to develop test systems to characterize the resistance of tumours against cytostatic agents (for review see [19]). Although some procedures have achieved clinical relevance in a few centres, no single test system has acquired widespread clinical acceptance and use. It is therefore not surprising that a general disillusionment has developed in this area of research.

In the last few years the concept of pleiotropic or multidrug resistance has been developed [20] and this phenotype of resistance has been extensively studied in animal and human cell systems in tissue culture. The understanding of the biological basis of multidrug resistance has begun to emerge through the application of molecular probes and monoclonal antibodies [21–26]. Nevertheless, up to now the data on human solid tumours with inherent or acquired resistance is sparse and up to now it is unclear whether the overexpression of the P-glycoprotein is indeed responsible for the resistance of these tumours. Because the estimations of multidrug gene products by Western- and Northern-blotting are time-consuming and the proportion of positive cases is very low, we used for the analysis the recently developed very sensitive phycoerythrin–streptavidin immunofluorescence method and the antibodies 265/F4 and C219, respectively. In the present study we have investigated the intrinsic resistance of a panel of four human epidermoid lung cancer xenografts grown in nude mice. The therapeutic responses of these tumours to cytostatic agents are precisely detectable and, as expected, the tumour lines responded differently to chemotherapy. When the expression of P-glycoprotein was correlated with the degree of resistance a close relationship could be demonstrated.

Bell *et al.* [4], using Western blot analysis, detected the P-glycoprotein in two out of five patients with pretreated solid ovarian cancer. In the present study we analysed the distribution of P-glycoprotein in not previously treated ovarian carcinomas. We found that one out of five ovarian carcinomas revealed a higher expression of P-glycoprotein while the other four ovarian carcinomas showed a lower level of expression. Using Western-blotting we found in all ovarian carcinomas bands

with M_r 170 kD but in one out the five tumours a higher expression of the P-170 protein. In addition, we determined the expression of genes responsible for multidrug-resistance by Northern-blotting with the pcDR 1.5 clone (kindly provided by Dr. Croop, Cambridge, MA, USA). An elevated level of RNA was detected only in the one ovarian carcinoma which showed an intensive immunofluorescence reaction (data not shown). Unfortunately for this study, after surgery all five patients were treated with cytostatic agents which are not involved in the multidrug resistance pattern. Therefore, it is not possible to carry out a connection between the degree of resistance and the degree of expression of P-glycoprotein.

Human leukaemias and plasmocytomas represent suitable models for acquired resistance. The present investigation indicates that two plasmocytomas and one leukaemia expressed high levels of P-glycoprotein, whereas two leukaemias showed moderate, and three leukaemias no expression of this protein. The correlation of the level of expression of P-glycoprotein in leukaemias with the clinical data is still superficial and more data will be needed in the future. The therapeutic regimens of patients with leukaemia of this study were so varied that an additional evaluation of clinical multidrug resistance was not possible.

In this investigation we used the recently developed, very sensitive, phycoerythrin–streptavidin immunofluorescence method. The biotinylation of label molecules (e.g. peroxidase and phycoerythrin) leads to a signal amplification by virtue of the ability of streptavidin to bind four biotin molecules. The formation of biotinylated phycoerythrin–streptavidin network complexes provides a very sensitive method which is convenient for the detection of antigens expressed at low level such as P-glycoprotein.

According to the data obtained in our investigation and the data of other authors the P-glycoprotein could eventually become both a prognostic tool to indicate resistant human tumour cells and a target molecule for chemotherapy to eliminate resistant cells of the human tumours. However, before such approaches are possible a greater knowledge of the distribution and of the relation to the degree of resistance in different human tumours is required.

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