Vol. 180, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1991 Pages 334-341

ANTIGENIC PROPERTIES ASSOCIATED WITH VINCA ALKALOID RESISTANCE IN OVARIAN CANCER CELLS: IDENTIFICATION OF A 92,000 DA PROTEIN [1]

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Received August 26, 1991

SUMMARY In order to characterize the membrane changes related to Vinca alkaloid resistance, we raised monoclonal antibodies (mAbs) against a Vincristine resistant subline (OV1/VCR) derived from a human ovarian adenocarcinoma cell line (OV1/p). Among three monoclonal antibodies selected for a higher binding to OV1/VCR than to OV1/p cells, one designated OVR09, recognized a Mr 92,000 protein. This protein appears to be gradually overexpressed along the drug resistance establishment *in vitro*, and to decrease slowly in absence of drug. Further, mAb OVR09 showed a much higher binding to the vinblastin resistant epidermoid tumor cell line KbV1 than to its parental counterpart. The Mr 92,000 protein was also detected in various tumor cell lines and in an ovarian carcinoma surgical sample.

Resistance to chemotherapeutic agents remains a major problem in the cure of cancers. Many studies have shed light on the 170-kDa transmembrane glycoprotein or P-glycoprotein, encoded by the MDR1 gene, which confers the multidrug-resistance (MDR) phenotype through an energy-dependant mechanism (1,2). At the membrane level, mechanisms of resistance specific to a class of drugs may coexist with increased amount of P-glycoprotein. For example, the overexpression of distinct membrane proteins have been reported in doxorubicin (DXR)-resistant cell line (3-5). From a parental human ovarian carcinoma cell line OV1/p (6), a vincristine-resistant cell line, namely OV1/VCR (7), has been obtained by drug stepwise selection. This resistant cell line exhibited a multidrug-resistant phenotype with increased levels of MDR1 gene transcripts, but this expression did not reflect the high relative index of OV1/VCR. In contrast, DXR-resistant subline (OV1/DXR) exhibited a typical MDR phenotype (Bénard *et al.*, in preparation).

[1]This work was supported by a grant from the Fondation M.M. Costes.

[2]Recipient of a research Fellowship from the Fondation M.M. Costes, Villejuif and the Société Française de Cancérologie, Paris.

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0006-291X/91 $1.50

Together these results suggested that drug resistance in OV1/VCR cells involved mechanisms other than those related to P-glycoprotein.

In an attempt to characterize membrane components associated with the Vinca alkaloid resistance phenotype, we studied the antigenic properties of OV1/VCR in comparison to those of OV1/p and derived sublines resistant to DXR (OV1/DXR) and cis-diammino dichloro platinum (OV1/DDP). Monoclonal antibodies (mAbs) were selected and one identified a 92,000 Da protein whose expression was analyzed during resistance induction/selection as well as on various cancer cell lines.

MATERIALS AND METHODS

Cell Culture. Cell lines were cultured in either DMEM or RPMI 1640 (GIBCO-BRL, Cergy-Pontoise, France) supplemented with 10% fetal calf serum (FCS). From OV1/p (6), various drug resistant sublines were derived by an *in vitro* drug stepwise procedure using independently VCR, DXR and cis-diammino dichloro platinum (CDDP) (8). Human epidermoid cell line KB 3-1 and two derived sublines, KB-8-5 (9) and KB-V1 (10), were kindly provided by M. Gottesman (NIH, Bethesda, MD). MCF7 and MCF7/DXR cell lines (11) were gifts of K. Cowan (NCI, Bethesda, MD).

Immunization and mAb production. Monolayer cell cultures of OV1/VCR cell line were washed with PBS and scraped with a rubber-policeman. Six-weeks-old BALB/c mice were immunized with $2.5x10^6$ OV1/VCR cells i.p. in 50% Freund complete adjuvant. Subsequent immunizations were performed as follows: 2.5×10^6 cells were injected monthly i.p. in 50% Freund incomplete adjuvant and a final boost was carried out by i.v. infusion of 10^5 cells, 3 days prior to cell fusion.

Selection of anti-OV1/VCR mAbs. Hybridomas producing anti-OV1/VCR antibodies were identified by using an indirect RIA carried out in 96-wells filter-bottomed microtitration plates (V&P Scientific, San Diego, CA). Plates were blocked by a 30 minutes incubation with FCS and then washed three times with PBS containing 10% FCS. OV1/VCR or OV1/p cells (10^5) in FCS were added to each well. The culture supernatants were added and incubated for 2h at room temperature. Cells were then washed and incubated for 2h with 10^5 cpm of [^{125}I]sheep anti-mouse Ig F(ab')$_2$ (Amersham, Les Ulis, France) diluted in PBS/50% FCS. After washing, radioactivity bound to filters was counted. The signal to noise ratio (S/N) was defined as the ratio of specific binding to non-specific binding, i.e., the binding of culture supernatant of the myeloma cells (NS1). MAbs presenting a higher binding to OV1/VCR than to OV1/p cells were selected. Purification, isotype determination and radioiodination of mAbs were performed as already described (12, 13).

Relative Resistance index was determined through an assay using {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)} (Sigma, La Verpillière, France) to determine the drug activity on proliferation. 2.10^4 cells were grown without drug on 24-wells culture plates for 48h and then incubated 24h with drug concentrations ranging from 1pg/ml to 50 µg/ml. Cells were then harvested by trypsinization and subsequently incubated in 96-wells culture plates for 96 h in absence of drug. MTT (0.5mg/ml) was added to each well and incubated for 4h at 37°C. Cells were then washed and incubated 10 minutes in pure DMSO. Absorbance was read at 540 nm and percentage of viable cells estimated (14). The relative resistance index was determined by the ratio of the drug concentration resulting in 50% survival of resistant and sensitive cells, respectively.

OVR09 mAb binding to cell lines. [^{125}I]OVR09 (10^5 cpm) were incubated with 10^5 cells in 200 µl of PBS/50% FCS for 2 h at room temperature. After washing three times, the radioactivity of dried filters was determined. Binding of mAbs to the various cell lines was also assessed on adherent cells. Cells were cultured in 24-wells culture plates (3 x 10^4 cells per well) for 2 days and then incubated for 2 hours at room temperature with 200 µl of [^{125}I]OVR09 (10^5 cpm). After 3 washes, cells were harvested and bound radioactivity was measured. Non specific binding was assessed by adding a large excess of unlabeled purified OVR09 mAb in the incubation test.

Western Blot Analysis. Confluent cells were harvested from culture flasks, washed twice in PBS, and resuspended in ice-cold PBS containing 0.01% aprotinin and 0.5% NP 40. After 1h of incubation at 4°C, lysates were centrifuged at 10,000 x g for 20 min at 4°C. Samples (1 mg/ml) were diluted in a 25 mM Tris-HCl buffer (pH 8.8) containing 0.1% SDS and 20% glycerol and then heated for 3 min at 95°C. About 50 µg of protein were analyzed through SDS-PAGE (15). Proteins were transferred electrophoretically to Polyvinylidene Fluoride (PVDF) membranes (Millipore, Saint-Quentin, France) which were then incubated at room temperature for 1h in PBS containing 5% milk and then with [^{125}I]OVR09 (2 x 10^5 cpm). Immunoreactive proteins were detected by autoradiography as previously reported (16). Reducing conditions were obtained by adding 2-mercaptoethanol (2%) to the sample solution.

RESULTS

Monoclonal Antibodies directed to OV1/VCR Cells. Among 1,068 antibody-secreting hybridomas produced against OV1/VCR cells, 25 clones secreting antibodies less reactive with the sensitive OV1/p cell line were selected. Three hybridomas expressed antibodies which presented a binding ratio (OV1/VCR versus OV1/p) greater than 3, namely OVR09, OVR11, and OVR12. Affinity constant of OVR09 mAb (IgG_{2a}) appeared to be similar on both OV1/VCR and OV1/p cells (3,3 x 10^9 M^{-1}) whereas antigenic determinants per cell were about 10^5 for OV1/VCR cells and 2 x 10^4 for OV1/p cells (ratio = 5). OVR11 mAb was found to be reactive with a protein also recognized by a mAb raised against trophoblastic cells (data not shown). Antigens identified by OVR11 and OVR12 are currently under investigation.

Expression of the antigen recognized by OVR09 during selection of resistant OV1/VCR cells. The binding of [^{125}I]OVR09 mAb was analyzed on cells corresponding to various passages during establishment of resistance to VCR of the OV1/VCR cell line (Figure 1). Our results showed an increase in antigen expression during the course of resistance induction/selection *in vitro* with a maximum after more than 320 cell generations and a resistance index of 800 (step 4). It was noteworthy that after about 100 generations in absence of drug, no significant change in the cell binding of OVR 09 mAb was observed while a decrease of 50% was found after 300 generations. Interestingly the same evolution was observed for the resistance index in absence of VCR with a similar decrease of resistance index.

Reactivity of OVRO9 mAb with various drug-resistant cell lines. [^{125}I]OVR09 mAb was found to bind significantly to OV1/VCR cells but weakly to both OV1/DDP and OV1/DXR cells (Table 1). A significant binding was also detected on a vinblastin resistant cell line (KB-V1) whereas the parental sensitive cell line (KB 3-1) and the

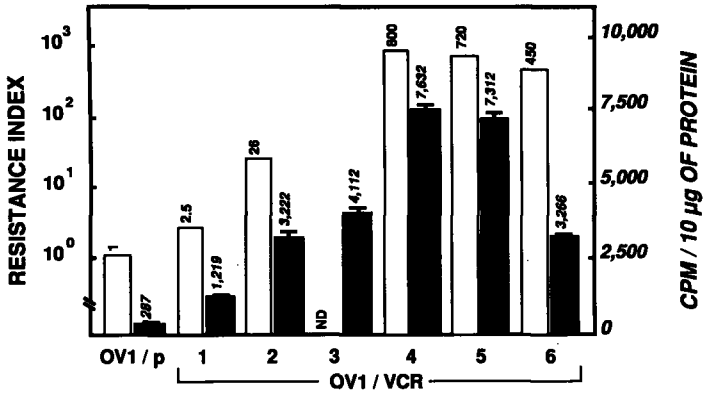

Figure 1. Comparison of [125I]OVR09 binding to resistance index during the course of resistance induction. [125I]OVR09 binding (■) was performed on OV1/VCR membrane preparation as described in "Materials and Methods". The level of resistance to VCR (□) was assessed by a clonogenic assay using MTT on the various isolates of OV1/VCR cells grown in the presence of VCR (*1*: 2 10^{-3}µg/ml for 6 passages; *2*: 2 10^{-3}µg/ml for 23 passages; *3*: 2 10^{-1}µg/ml for 6 passages; *4*: 2 10^{-1}µg/ml for 78 passages) and in the absence of VCR (*5*: after 23 passages; *6*: after 67 passages). In our culture conditions, 1 passage represent about 4 cell generations. OV1/p denotes the parental sensitive cell line.

colchicine resistant cell line (KB-8-5) did not overexpress the antigen. Surprisingly, the MCF7/DXR cell line presented a greater immunoreactivity than the sensitive parental cell line.

Cell distribution of the antigen defined by OVRO9 mAb. The reactivity of [125I]OVR09 mAb to various cell lines was examined using the cell binding radioimmunoassay. Results showed that the antigen was present on some cell lines derived from hepatomas (Hep 3B, HUH 7, Hep G2, PLC/PRF/5), choriocarcinoma (JEG-3), uterin mixed mesodermal tumor (SK-UT-1) and

Table 1. 125I-OVR 09 binding to various drug-resistant and sensitive cell lines

Cell line	Drug of selection	Selection conc. (µg/ml)	Resistance Index	Reactivity with OVR 09 (cpm) [a]	Ratio Res/Sens [b]
OV1/p			1	1,230	1.0
OV1/VCR	Vincristine	0.20	800	6,260	5.0
OV1/DXR	Adriamycin	0.10	20	850	0.7
OV1/DDP	Cisplatin	1.00	10	800	0.7
KB 3-1			1	45	1.0
KB 8-5	Colchicine		4	110	2.4
KB V1	Vinblastin	1.00	213	1,240	27.5
MCF7			1	300	1.0
MCF7/DXR	Adriamycin	6.00	192	1,020	3.4

[a] Direct binding measured on adherent cells (100,000 cpm/50,000 cells/well). The indicated values represent the mean of triplicate samples. The SDs values were all less than 10% of the sample mean.

[b] Res/Sens: binding ratio of resistant versus parental cell line.

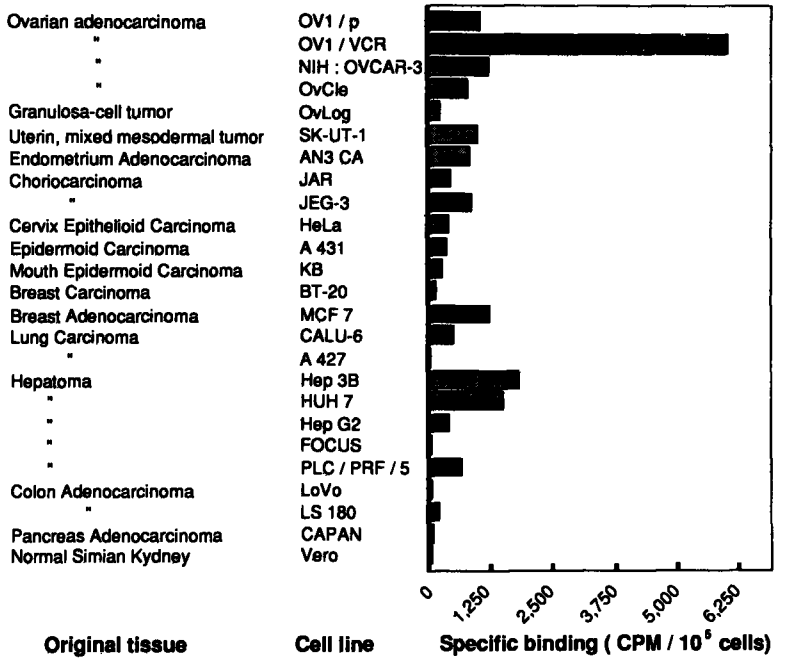

Figure 2. [^{125}I]OVR09 mAb binding to antigens expressed on various tumor cell lines. Experiments were performed on microtitration plates as described in "Materials and Methods".

adenocarcinomas of breast (MCF7) and endometrium (AN3CA) (Fig. 2). Other lines tested were either less or non reactive with [^{125}I]OVR09 mAb.

Identification of the antigen recognized by OVRO9 mAb. Western-immunoblot analysis performed under non-reducing conditions showed that OVRO9 mAb recognized a single large band at a M_r 92,000 in OV1/VCR cell extract while it was weakly detected in the parental OV1/p cells. The same band was observed in an antigenic extract prepared from an ovarian carcinoma specimen, OVT 01, obtained from a patient undergoing surgery (Fig. 3). KB-V1 and HUH7 cell lines appeared to express the same M_r 92,000 protein whereas MCF7 presented an additional slightly stained band at 50,000 Da (data not shown). The M_r 92,000 band was not detectable after reducing or trypsin treatments on OV1/VCR, indicating that the antigenic site recognized by the OVR09 mAb is dependent on a tertiary stucture of the protein and sensitive to proteolytic activities.

DISCUSSION

In the present report, we investigated some of the antigenic changes associated with pleiotropic drug-resistance development in cancer cells in studying a VCR-resistant cell line. Several mAbs were selected according to their respective binding to resistant and sensitive cells. From 9 fusion experiments, 3 mAbs, namely OVR09, OVR11 and OVR12, displayed a higher binding to resistant cells than to sensitive ones. We focused mainly on the characterization of an antigen recognized by the

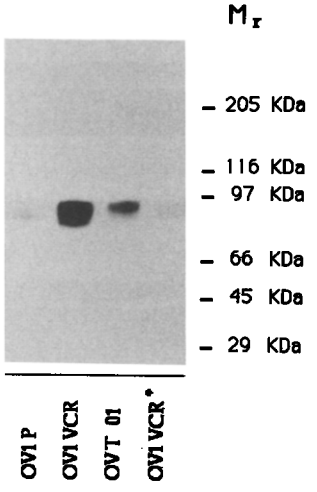

Figure 3. Characterization of the antigen recognized by OVR09 mAb by Western immunoblotting. About 50 μg of protein was loaded on a 10% SDS-polyacrylamide gel. OVT01 denotes a NP40 extract prepared from a fresh-frozen ovarian carcinoma tumor. OV1/VCR* denotes a 2-mercaptoethanol treated sample of OV1/VCR. Immunoblot was carried out as described in "Materials and Methods".

OVRO9 mAb. The features of this antigen are: (a) a change in antigen expression during the course of resistance establishment *in vitro,* (b) its overexpression in two different Vinca alkaloid resistant cell lines as compared to the sensitive cell lines, (c) its location on a cell surface protein with a M_r of 92,000 Da that is trypsin-sensitive, (d) its distribution on some tumor cell lines, though the binding of OVR09 mAb was lower than to the OV1/VCR cell line.

The pharmacological and molecular bases for the typical multidrug-resistant (MDR) phenotype expressed by cells selected by continuous stepwise exposure to drug are now well-documented (17). Unlike cells which display this classic MDR phenotype, unusual cross-resistance pattern has been described in various drug-resistant cell lines viewed as non "typical" MDR (18). Investigation of alternate and/or complementary mechanisms to P-glycoprotein at the cell membrane level is particularly challenging. VCR resistant human promyelocytic leukemia cells, HL60, contain increased levels of both P-glycoprotein and a distinct M_r 150,000 protein (p150) (19). Such p150 is also present in HL60 cells isolated for resistance to Adriamycin that are devoid of P-glycoprotein. Minski and Cole selected 6 mAbs which react specifically with the multidrug-resistant H69AR cell line, which does not overexpress P-glycoprotein (20). One of them reacts with a cell surface membrane-associated antigens with molecular weights between 24.500 and 34.500. Tsuruo and his group reported the characterization of two antigens specifically expressed in DXR-resistant human tumor cells, one being expressed on a membrane M_r 85,000 protein concomitantly with P-glycoprotein (3,4). Recently, a surface membrane protein with an apparent M_r 95,000 was also reported in a DXR-resistant cell line which does not overexpress P-glycoprotein (5). Of particular interest is the

finding that acquired clinical drug-resistance possibly appears to be an atypical form of multidrug resistance (21, 22).

Expression of the antigen recognized by OVRO9 appears to be associated with the pulse Vinca alkaloid treatment of cells *in vitro*, suggesting that the protein might contribute to drug resistance in these cells. Although strong evidence for this contribution is difficult to obtain since cells also contain high levels of P-glycoprotein, it is noteworthy that (a) a correlation exists between the drug-resistance index and the expression of the antigen and (b) the expression of the M_r 92,000 protein which appears early in the resistance establishment and decreases slightly after a long period of growth in the absence of VCR. It remains to be determined whether the M_r 92,000 protein is directly involved in drug-resistance or represents a resistance-associated epiphenomenon. In the first case, the antigen might contribute for an unknown part to the resistance development. Indeed, OV1/VCR cells display both MDR1 gene overexpression without genomic DNA amplification and a high relative resistance index (6). This non "typical" MDR feature might be accounted for by the discontinuous drug-exposure used for the *in vitro* selection, which mimics the tumor treatment in patients, resulting in a particular antigenic phenotype.

Further studies are necessary to determine the clinical interest of the antigen recognized by OVR09 mAb. Preliminary immunohistochemical results demonstrated that this antigen is weakly expressed in human normal ovarian tissue but is overexpressed in some epithelial adenocarcinomas of the ovary (T. Voeltzel *et al.*, unpublished results). cDNAs cloning experiments are in progress and might be useful for investigating the functional role, if any, of this M_r 92,000 protein.

ACKNOWLEDGMENTS

The authors wish to thank Drs. M. Gottesman and K. Cowan for kindly providing us with drug-resistant and sensitive cell lines, Drs. M. Ozturk and J. Wands for hepatoma cell lines and helpful discussions and D. Costes for continuous support. We also thank C. Bombled and C. Potentini for expert technical assistance.

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