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Efficient inhibition of P-glycoprotein-mediated multidrug resistance with a monoclonal antibody

(*MDR1* gene/cancer chemotherapy/immunotherapy/membrane transport)

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ABSTRACT **P-glycoprotein (Pgp), encoded by the *MDR1* gene, is an active efflux pump for many structurally diverse lipophilic compounds. Cellular expression of Pgp results in multidrug resistance (MDR) *in vitro* and is believed to be a clinically relevant mechanism for tumor resistance to chemotherapy. We have developed a mouse monoclonal antibody, UIC2, that recognizes an extracellular epitope of human Pgp. UIC2 inhibited the efflux of Pgp substrates from MDR cells and significantly increased the cytotoxicity of Pgp-transported drugs, under the conditions where no effect was detectable with other anti-Pgp antibodies. Potentiation of cytotoxicity by UIC2 was observed with all the tested drugs associated with MDR (vinblastine, vincristine, colchicine, taxol, doxorubicin, etoposide, actinomycin D, puromycin, and gramicidin D) but not with any of the drugs to which MDR cells are not cross-resistant (methotrexate, 5-fluorouracil, *cis*-platinum, G418, and gentamicin). The inhibitory effect of UIC2 *in vitro* was as strong as that of verapamil (a widely used Pgp inhibitor) at its highest clinically achievable concentrations. Our results suggest that UIC2 or its derivatives provide an alternative or supplement to chemical agents for the reversal of MDR in clinical cancer.**

P-glycoprotein (Pgp), the product of the *MDR1* gene, is a transmembrane protein that carries out energy-dependent efflux of many structurally diverse lipophilic compounds, including many anticancer agents (1). Pgp-mediated multidrug resistance (MDR) appears to be an important clinical component of tumor resistance to chemotherapy, judging from the high incidence of *MDR1* expression in tumors of various types (2, 3) and the observed correlations between *MDR1* expression and lack of response to chemotherapy in various types of cancer (4, 5).

The establishment of the role of the *MDR1* gene in the resistance to clinically useful drugs has prompted a major effort aimed at finding agents capable of reversing *MDR1*-mediated MDR. Many classes of chemicals, including verapamil and some other calcium channel blockers, cyclosporins, steroid hormones, and other compounds, were found to enhance the intracellular accumulation and cytotoxic action of Pgp-transported drugs (6). Many Pgp inhibitors themselves bind to and are pumped out of the cell by Pgp (7–9). Some of these agents may have other effects on drug transport or cytotoxicity in MDR cells, aside from the inhibition of Pgp-mediated efflux (10, 11). Furthermore, MDR-reversing agents used in the initial clinical trials have major side effects unrelated to the inhibition of Pgp, such as cardiac toxicity (verapamil) or immunosuppression (cyclosporin A), that limit their clinically achievable concentrations.

The potential utility of antibodies recognizing Pgp on intact cells as specific agents for the reversal of MDR was first suggested by the work of Hamada and Tsuruo (12). These investigators found that treatment of some MDR cell lines with a monoclonal antibody (mAb) MRK16 increased intracellular accumulation and cytotoxicity of vincristine and actinomycin D. MRK16 also increased the *in vivo* toxicity of vincristine to a human MDR cell line grown as a xenograft in nude mice (13). The *in vitro* potentiation of drug cytotoxicity by MRK16, however, was weak relative to that of chemical inhibitors and was apparently limited to only some of the Pgp substrates, since MRK16 had no effect on doxorubicin cytotoxicity (12). An increase in the accumulation of vincristine and actinomycin D was also observed with two other anti-Pgp mAbs, HYB-241 and HYB-612 (14), but no effects of these antibodies on *in vitro* drug cytotoxicity have yet been reported, to our knowledge.

In the present study, we report the isolation and characterization of a mAb (UIC2) that recognizes human Pgp on the surface of intact cells. UIC2 strongly inhibits Pgp-mediated efflux and the resistance of MDR cells to all the tested cytotoxic drugs transported by Pgp. The inhibitory effect of this mAb was as strong as that of verapamil at its highest clinically achievable concentrations. These results indicate that UIC2 or its derivatives may provide an alternative or supplement to chemical agents in the reversal of MDR in clinical cancer.

MATERIALS AND METHODS

MDR Cell Lines. MDR derivatives of mouse BALB/c 3T3 fibroblasts were generated by transfection with human MDR1 cDNA in a mammalian expression vector pUCFVXMDR1 (15), followed by a multistep selection of transfectants for resistance to vinblastine at 1 μg/ml. The resulting cell line was designated BALB/c 3T3-1000 (K. Choi and I.B.R., unpublished data). The K562/Inf cell line was derived by infection of human K562 leukemia cells with a recombinant retrovirus pLMDR1L6 carrying human MDR1 cDNA (16) and subsequent subcloning, without cytotoxic selection (P. M. Chaudhary and I.B.R., unpublished data). LRMN1 cell line was obtained by transfection of Chinese hamster LR73 fibroblast cells with a plasmid pUCFVXMDR1/neo that expresses human MDR1 cDNA and the neo (G418 resistance) gene (B. Morse and I.B.R., unpublished data), followed by selection with G418 (D. de Graaf and I.B.R., unpublished data). MDR cell lines KB-8, KB-8-5, KB-8-5-11, and KB-V1, isolated from human KB-3-1 carcinoma cells by multistep selection with colchicine (17) or vinblastine (18), were obtained from M. M. Gottesman (National Cancer Institute, Bethesda, MD). KB-GRC1 cells were

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Abbreviations: Pgp, P-glycoprotein; MDR, multidrug resistance (resistant); mAb, monoclonal antibody; Rh123, rhodamine 123; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide.

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derived from KB-3-1 by transfection with pUCFVXMDR1 and colchicine selection (15). CEM/VLB_{100} cell line, derived from human CEM leukemia cells by multistep selection with vinblastine (19), was provided by W. T. Beck (St. Jude's Childrens Hospital, Memphis, TN). Vinblastine- or colchicine-selected MDR derivatives of mouse J774.2 macrophage cell line, J7-V2-1, J7-V3-1, and J7-C1-100 (20), were a gift of S. B. Horwitz (Albert Einstein College, Bronx, NY).

Antibodies. mAb MRK16 (IgG2a) against human Pgp (12) was a gift of T. Tsuruo (University of Tokyo). Anti-Pgp mAbs HYB-241 and HYB-612 (both IgG1) (14) were obtained from L. Rittmann-Grauer (Hybritech), and mAb C219 (IgG2a) (21) was purchased from Centocor (Malvern, PA). Affinity-purified preparations of mouse myeloma protein UPC10 (IgG2a) or mouse IgG were obtained from Sigma. All mAb samples were at least 95% pure IgG by SDS/PAGE. When necessary, mAbs were extensively dialyzed against phosphate-buffer saline (PBS) or Dulbecco's modified Eagle's medium (DMEM).

Development of mAb UIC2. Hybridomas were developed from BALB/c mice immunized with BALB/c 3T3-1000 cells by standard procedures (22). Hybridoma supernatants were screened by indirect immunofluorescence microscopy of live BALB/c 3T3 and BALB/c 3T3-1000 cells. One hybridoma clone of 556 clones tested produced a mAb, termed UIC2, which was reactive with BALB/c 3T3-1000 but not with the parental BALB/c 3T3 cells. mAb UIC2 was purified by protein A (Bio-Rad) column chromatography, according to manufacturer's instructions. The isotype of mAb UIC2 was determined with mouse IgG subclass-specific antisera (ICN). The ability of mAb UIC2 to induce complement-mediated cytotoxicity was tested using Low-Tox-M rabbit complement (Cedarlane Laboratories, Hornby ON, Canada).

Radioimmunoprecipitation. For radioimmunoprecipitation, 5–10 × 10^6 cells were metabolically labeled with [^{35}S]methionine (ICN) at 50 μCi/ml (1 Ci = 37 GBq) in methionine-free DMEM with 10% (vol/vol) fetal calf serum for 10–18 h. The cells were rinsed with PBS, and a purified mAb was added in 2.6 ml of PBS to a final concentration of 20 μg/ml. After a 1-h incubation at room temperature, the cells were rinsed with PBS and then lysed with a detergent solution containing 0.2% deoxycholic acid and 0.2 mM phenylmethylsulfonyl fluoride (Sigma) in PBS for 2–4 min. The lysates were precleared by microcentrifugation for 15 min at 4°C and incubated with immobilized protein A beads (Repligen, Cambridge, MA) for 1 h at 4°C. After five washes with the lysis solution, the precipitated protein was analyzed by SDS/PAGE in 7.5% gels.

Flow Cytometric Assays. Indirect immunofluorescence labeling, dye efflux assays and flow cytometry were carried out essentially as described (23). To study the effect of mAbs on the efflux of fluorescent compounds, 10^6 suspended cells were incubated with various mAbs at 20 μg/ml in 3–5 ml of serum-free medium for 30 min on ice and then washed twice and loaded on ice with rhodamine 123 (Rh123) at 0.5–1.0 μg/ml for 10 min or with 5 μM doxorubicin for 1 h. mAbs (20 μg/ml) were also added to dye-free medium during the efflux period at 37°C. In absorption experiments, purified mAb UIC2 at 20 μg/ml was preincubated with 0.3 ml of agarose beads (Sigma) coupled with affinity-purified goat anti-mouse IgG or anti-mouse IgM for 1 h on ice, and the absorbed material was removed by centrifugation. UPC10 (IgG2a) was used as the isotype control for mAbs UIC2 and MRK16; mouse IgG was included as a control for mAbs HYB-241 and HYB-612.

Drug Cytotoxicity Assays. All the drugs were obtained from Sigma, except for G418 (GIBCO) and taxol (a gift of S. B. Horwitz). Colony formation and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assays for cell growth inhibition were carried out as described (15, 24). The effect of mAbs on drug cytotoxicity was assayed by incubating cells growing in suspension or trypsinized monolayer cells with purified mAbs, predialyzed against DMEM, for 30 min at room temperature. DMEM with 10% (vol/vol) heat-inactivated fetal calf serum was used in all experiments. Cells were plated in triplicate in 6-well tissue culture plates (Falcon; 200–250 cells per well for colony assays and 400–450 cells per well for MTT assays) in the presence of mAbs. K562/Inf cells were grown for MTT assays in 96-well plates in quadruplicate.

RESULTS

Characterization of mAb UIC2. The reactivity of mAb UIC2 (IgG2a) with the human *MDR1* gene product was initially determined by immunofluorescence staining of various MDR cell lines (Table 1). UIC2 reacted with all the tested cell lines that expressed the human *MDR1* gene, including human or rodent cells transfected with human MDR1 cDNA and isolated with or without cytotoxic selection, as well as with CV1-COS green monkey kidney cells known to express Pgp (25), but not with any Pgp-negative cell lines. UIC2 binding correlated with the known levels of drug resistance in the cell lines tested. UIC2 failed to react with MDR derivatives of mouse J774.2 cells (20) that expressed Pgps encoded by each of the three mouse *mdr* genes.

Radioimmunoprecipitation (Fig. 1) showed that mAb UIC2 recognizes a single diffuse band of 170–180 kDa that was present in MDR cells and comigrated with the band that was immunoprecipitated by the Pgp-specific mAb MRK16 (12). Immunoprecipitation by UIC2 required solubilization of membrane proteins in a deoxycholate-containing buffer; solubilization with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (26) allowed for efficient immunoprecipitation of Pgp by MRK16 but not by UIC2 (data not shown). UIC2 did not react with denatured Pgp on Western blots under the conditions where Pgp was detectable with another mAb (C219; data not shown).

Inhibition of Pgp-Mediated Efflux of Fluorescent Dyes. We have used a flow cytometric assay to investigate the effect of mAb UIC2 on Pgp-mediated efflux. In this assay (23, 27), MDR cells were stained at 0°C with a mitochondrial fluores-

Table 1. Reactivity of mAb UIC2 with MDR cell lines

Cell line	UIC2 reactivity
Human, selected for MDR	
KB-3-1 (parent)	−
KB-8	±
KB-8-5	+
KB-8-5-11	++
KB-V1	+++
CEM (parent)	−
CEM-VLB-100	++
Transfected with human MDR1 cDNA	
K562 (recipient)	−
K562/Inf	+
BALB/c 3T3 (recipient)	−
BALB/c 3T3-1000	+++
LR73 (recipient)	−
LRMN1	+
Green monkey kidney (CV1-COS)	+
Mouse, selected for MDR	
J774.2 (parent)	−
J7-V2-1	−
J7-V3-1	−
J7-C1-100	−

UIC2 reactivity, evaluated by a flow cytometric assay, is expressed as relative values, from − (no detectable reactivity) to +++ (highest reactivity).

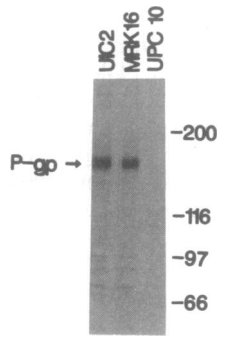

FIG. 1. Radioimmunoprecipitation of Pgp. mAbs used are indicated. The 170- to 180-kDa band specific to mAbs UIC2 and MRK16 is indicated by an arrow. Molecular masses of size standards (in kDa) are shown to the right.

cent dye Rh123 and then incubated in dye-free medium at 37°C, during which time Pgp pumps Rh123 out of the cells. We have found that UIC2 at 20 μg/ml (mAb concentration found to be saturating for all the tested cell lines) inhibited Rh123 efflux from MDR cell lines CEM-VLB-100 and K562/Inf, relative to control cells treated with an unrelated isotype-matched antibody (UPC10) (Fig. 2). The effect of UIC2 was abolished after preabsorption with anti-mouse IgG but not with the control anti-mouse IgM sorbent (data not shown). In contrast to UIC2, three other mAbs recognizing human Pgp on the cell surface, including MRK16 (Fig. 2), HYB-612, and HYB-241 (data not shown), showed no inhibition of Rh123 efflux, even though all four antibodies were used at the same saturating concentration and stained all the tested MDR cell lines with the same intensity. UIC2 also decreased the efflux of a fluorescent anticancer drug, doxorubicin, from MDR cells (data not shown).

Reversal of Resistance to Pgp-Transported Drugs. We have investigated the effect of UIC2 on the cytotoxicity of Pgp-transported drugs to MDR cells. As shown in Fig. 3*A*, the addition of mAb UIC2 strongly potentiated the growth inhibition of BALB/c 3T3-1000 cells by vinblastine to decrease its ID_{50} value from 650 ng/ml to 150 ng/ml. In the absence of

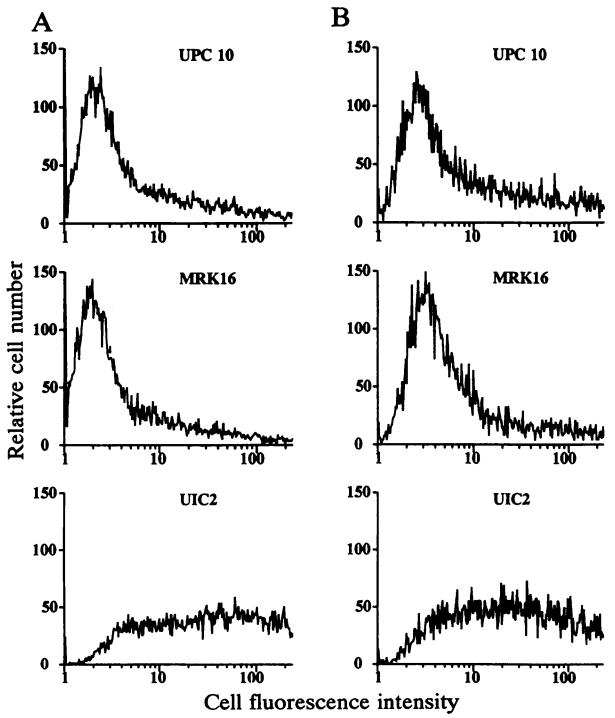

FIG. 2. Effect of UIC2 on efflux of fluorescent Pgp substrates from MDR cells. (*A*) CEM-VLB-100 cells were loaded with Rh123 at 10 μg/ml on ice, incubated in dye-free medium for 30 min at 37°C, in the presence of mAbs UIC2, MRK16, or UPC10, and analyzed by flow cytometry. (*B*) K562/Inf cells were loaded with Rh123 at 1 μg/ml on ice and incubated in dye-free medium for 40 min at 37°C with the same mAbs.

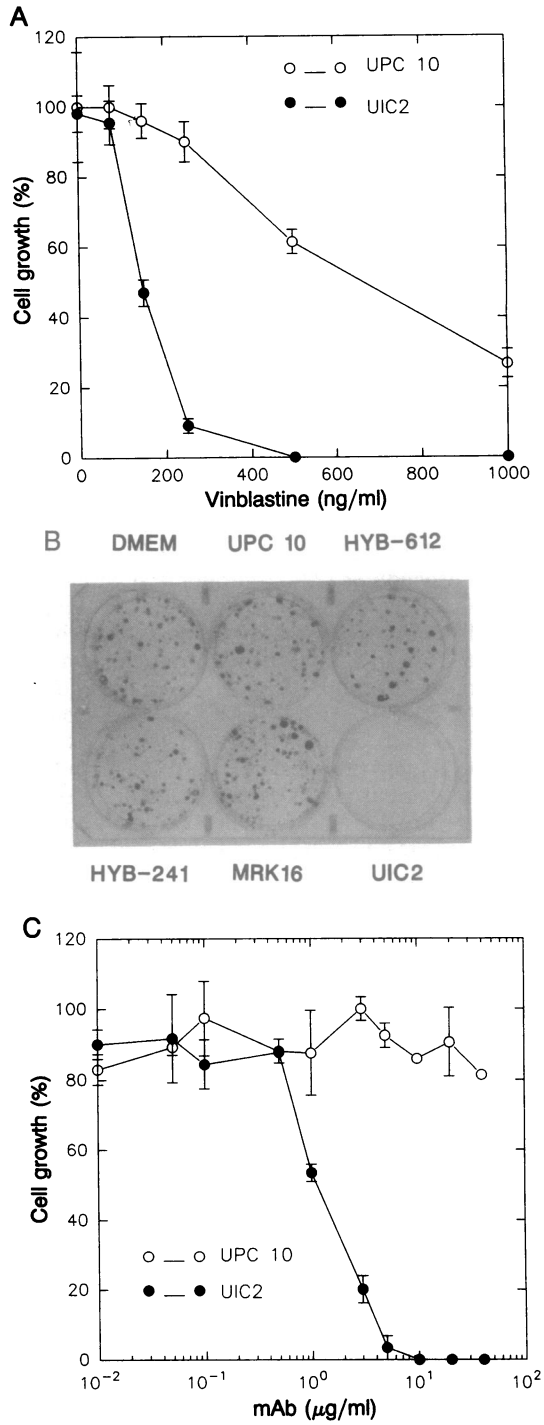

FIG. 3. Potentiation of vinblastine cytotoxicity by mAb UIC2. (*A*) Inhibition of BALB/c 3T3-1000 cell growth by various concentrations of vinblastine in the presence of mAb UIC2 or a nonspecific control UPC10 at 20 μg/ml. All the values are expressed relative to UPC10-treated BALB/c 3T3-1000 cells grown in the absence of the drug. All the assays were done in triplicate. (*B*) Effect of various mAbs on colony formation by BALB/c 3T3-1000 cells in the presence of vinblastine. About 200 cells per well were plated in the presence of vinblastine at 350 ng/ml (ID_{90}) and the indicated mAbs at 20 μg/ml. Colonies were stained with crystal violet on day 11. (*C*) Effect of various concentrations of mAb UIC2 on BALB/c 3T3-1000 cell growth in the presence of vinblastine at 350 ng/ml (ID_{90}) or in the absence of the drug. All the values are expressed relative to UPC10-treated BALB/c 3T3-1000 cells grown in the absence of the drug.

the drug, UIC2 had no effect on the growth of BALB/c 3T3-1000 cells. Fig. 3*B* shows that colony formation by BALB/c 3T3-1000 cells in the presence of vinblastine at 350 ng/ml (ID_{90}) was completely eliminated by UIC2 but not by the other mAbs against Pgp (MRK16, HYB-241, and HYB-612) or by an unrelated antibody (UPC10), used at the same saturating concentration. The potentiating effect of UIC2 on the cytotoxicity of vinblastine at 350 ng/ml in BALB/c 3T3-1000 cells became detectable with UIC2 at $\approx$1 μg/ml and led to complete suppression of cell growth at 10 μg/ml (Fig. 3*C*). Verapamil, a well-characterized chemical inhibitor of Pgp, achieved the same potentiation of vinblastine toxicity to BALB/c 3T3-1000 cells at 3 μM (data not shown). UIC2 also decreased the level of drug resistance in the other tested MDR cell lines, including K562/Inf, KB-GRC1, and KB-V1 (data not shown).

To determine whether the potentiating effect of UIC2 on cytotoxicity was limited to only some of Pgp-transported drugs, we have tested this effect using nine Pgp substrates, including vinblastine, vincristine, colchicine, taxol, doxorubicin, etoposide, actinomycin D, puromycin, and gramicidin D. At the ID_{50} concentrations of these drugs, the addition of UIC2 at 20 μg/ml greatly decreased the growth of BALB/c 3T3-1000 cells (Fig. 4). The inhibition of cell growth in UIC2-treated relative to control cells ranged from 100% for vinblastine, doxorubicin, actinomycin D, and taxol to 68% for colchicine. When a similar experiment was conducted at drug concentrations corresponding to ID_{20} values, the addition of UIC2 inhibited cell growth by 97–100% for all the tested drugs (data not shown). mAb UIC2 had no effect on the cellular response to ID_{50} doses of five cytotoxic drugs not transported by Pgp, including methotrexate, 5-fluorouracil, *cis*-platinum, G418, and gentamicin (Fig. 4). The cytotoxicity-potentiating effect of UIC2 is, therefore, specific to Pgp substrates.

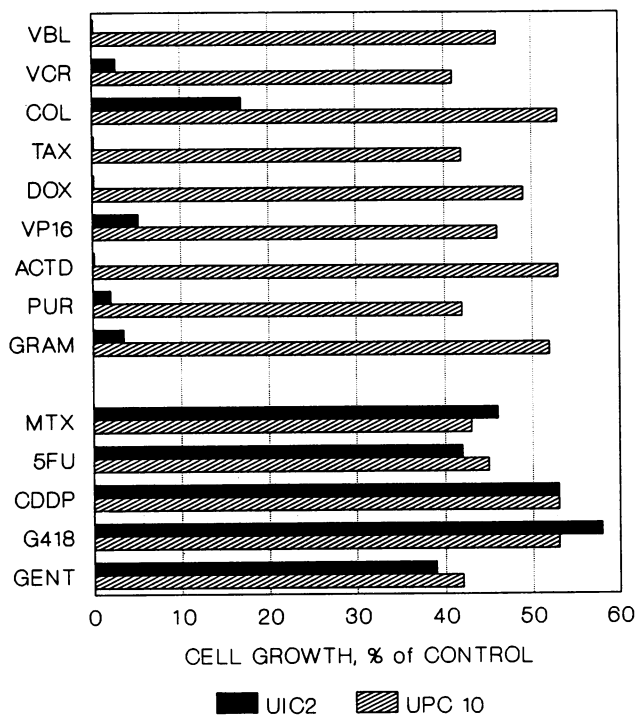

FIG. 4. Potentiation of cytotoxic effects of various drugs by mAb UIC2. Bars represent the viability of BALB/c 3T3-1000 cells (as determined by the MTT assay) in the presence of mAb UIC2 at 20 μg/ml (solid bars) or UPC10 control at 20 μg/ml (hatched bars). The mean of triplicate measurements is shown; standard deviation was <20% of each mean value. Cell viability is expressed relative to that of control cells grown with UPC10 in the absence of drugs. The drug concentrations, corresponding to predetermined ID_{50} values, were as follows: vinblastine (VBL), 0.73 μM; vincristine (VCR), 3.1 μM; colchicine (COL), 1 μM; taxol (TAX), 1.6 μM; doxorubicin (DOX), 0.4 μM; etoposide (VP16), 2.23 μM; actinomycin D (ACTD), 0.06 μM; puromycin (PUR), 37.5 μM; gramicidin D (GRAM), 4.1 μM; methotrexate (MTX), 0.04 μM; 5-fluorouracil (5FU), 0.46 μM; *cis*-platinum (CDDP), 0.17 μM; G418, 96 μM; gentamicin (GENT), 24 μM.

DISCUSSION

Despite multiple efforts, only a few mAbs reactive with extracellular epitopes of Pgp have been developed (12, 14, 28). mAb UIC2, isolated in the present study, belongs to the same category, judging from its ability to stain the surface of live cells expressing the human *MDR1* gene and to immunoprecipitate a single protein with the electrophoretic mobility of Pgp. UIC2 is apparently specific to the *MDR1* gene product, since it did not react detectably with tMDR3.35 cells (29) that express cDNA of the closely related human *MDR2* (*MDR3*) gene (A. Schinkel and P. Borst, personal communication). mAb UIC2 is likely to become a useful reagent for the diagnostics of Pgp expression and for the purification of Pgp-expressing cells.

Inhibition of multidrug resistance by mAb UIC2 was much stronger and qualitatively different than previously reported for other mAbs reactive with extracellular domains of Pgp. MRK16 (12), as well as HYB-241 and HYB-612 (L. Rittmann-Grauer, personal communication), selectively increased accumulation of vincristine and actinomycin D but not of doxorubicin. In contrast, the inhibitory effect of UIC2 was apparent with all the tested Pgp substrates. Furthermore, MRK16 was reported to cause only partial inhibition of cell growth at all the tested drug concentrations, and its effect was more pronounced at intermediate than at high drug concentrations (12, 13). This unusual dose dependence and drug specificity of inhibition suggest that MRK16 may act by a mechanism different from direct inhibition of Pgp-mediated drug efflux (30). In contrast, the inhibitory effect of mAb UIC2 was similar to that of chemical inhibitors, as it was more pronounced at the higher drug doses and led to complete cell killing at lower drug concentrations. In a functional assay, UIC2 directly inhibited Pgp-mediated efflux of Rh123 from MDR cells.

Under our experimental conditions, Rh123 efflux and vinblastine cytotoxicity were unaffected by saturating concentrations of MRK16, HYB-241, or HYB-612 mAbs. [Our failure to detect any effect of MRK16, HYB-241, and HYB-612 does not necessarily contradict the reports of other investigators (12–14), since we have tested these mAbs with a different set of Pgp substrates.] Recent results of A. Schinkel and P. Borst (personal communication) indicate that UIC2 recognizes a different epitope on Pgp than the other mAbs. Specifically, UIC2 failed to react with cells that expressed a mutant form of Pgp with a deletion within the first extracellular loop, whereas MRK16 and HYB-241 were reactive with the same cells. Since only UIC2 provided efficient reversal of MDR, it appears that Pgp inhibition by mAbs is epitope-specific.

Anti-Pgp mAbs have been used to destroy Pgp-expressing cells through immunotoxin conjugates (31) or through complement-mediated (32) or antibody-mediated cytotoxicity (33). A potential problem with clinical application of this approach is a toxic effect to Pgp-expressing normal tissues. An alternative strategy involves the use of anti-Pgp antibodies or their derivatives (antibody fragments, humanized antibodies, etc.) as noncytotoxic supplements to standard chemotherapeutic regimens. This strategy would be expected to provide a higher therapeutic ratio, since Pgp-inhibiting antibodies in such regimens would increase the intracellular accumulation of chemotherapeutic drugs that are more toxic

to tumor than to normal cells, rather than induce cytotoxicity to all Pgp-expressing cells.

Pgp-inhibiting mAbs may also have several advantages over chemical agents reversing MDR. (*i*) Unlike most chemical inhibitors, mAbs should have no associated toxicity other than that resulting from host immune reactions; the latter can be controlled by appropriate modification of mAbs. (*ii*) Increased accumulation of cytotoxic drugs in Pgp-expressing normal cells may be less pronounced with mAbs than with chemical inhibitors of Pgp. Thus, much of Pgp expression in normal tissues is associated with the luminal surfaces of the gastrointestinal tract and excretory organs (34). These surfaces would be more accessible to diffusable chemical compounds than to antibodies administered parenterally. (*iii*) Almost all of the characterized chemical agents reversing MDR are themselves transported out of cells by Pgp and, therefore, provide only transient inhibition. In contrast, mAbs would be expected to give rise to more prolonged inhibition due to the relatively stable nature of their binding to Pgp.

The weak functional effects of the previously reported anti-Pgp antibodies relative to chemical inhibitors of Pgp and their limited drug specificity have been the main concerns for the immunotherapeutic approach to the circumvention of MDR (30). In the present study, we found that mAb UIC2 significantly increased the cytotoxicity of all the tested Pgp substrates, including such clinically important drugs as doxorubicin, vinblastine, vincristine, actinomycin D, and taxol. UIC2 had maximal inhibitory effect at its saturating concentration, which was 10 μg/ml for cells expressing high levels of Pgp. This or higher plasma concentration can be readily achieved with the doses of mouse mAbs that have been administered to patients in clinical trials (35). The effect of UIC2 in BALB/c 3T3-1000 cells was equivalent to that of 3 μM verapamil, the modifier of MDR that was most extensively used in clinical trials. This is somewhat higher than the median peak serum concentration of verapamil achieved in a recent clinical study (2.24 μM; ref. 36). Our results indicate that mAb UIC2 or its derivatives are highly specific and potent inhibitors of Pgp-mediated MDR and may provide an alternative to chemical agents for the reversal of MDR in clinical cancer.

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