Brief Definitive Report

The Multidrug Resistance Phenotype Confers Immunological Resistance

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Summary

Multidrug resistance (MDR), which is due, in part, to the overexpression of P-glycoprotein, confers resistance to a variety of natural product chemotherapeutic agents such as daunorubicin, vincristine, and colchicine. RV^+ cells are a P-glycoprotein overexpressing variant of the HL60 myeloid leukemia cell line. In addition to classic MDR, RV^+ cells displayed relative resistance to complement-mediated cytotoxicity with both immunoglobulin G and M antibodies against different cell surface antigens, but not to antibody-dependent cellular cytotoxicity and lymphokine-activated killing. Complement resistance was reversed both by treatment with verapamil and with specific monoclonal antibodies (mAbs) capable of binding to P-glycoprotein and blocking its function. To further confirm that the resistance of RV^+ cells was not a consequence of the selection of the cells on vincristine, a second system involving P-glycoprotein infectants was also investigated. K562 cells infected with the MDR1 gene, which were never selected on chemotherapeutic drugs, also displayed relative resistance to complement-mediated cytotoxicity. This MDR1 infection–induced resistance was also reversed by mAbs that bind to P-glycoprotein. Therefore, the MDR phenotype as mediated by P-glycoprotein provides resistance to complement-mediated cytotoxicity. The increased intracellular pH and the decreased membrane potential due to the MDR phenotype may result in abnormal membrane attack complex function. This observation may have implications for the possible mechanisms of action of P-glycoprotein and for a possible physiologic role for P-glycoprotein in protection against complement-mediated autolysis.

Multidrug resistance (MDR) is characterized by broad resistance to several pharmacologically and chemically distinct chemotherapeutic compounds, in particular, the vinca alkaloids and the anthracyclines (1). Tumor cells that are resistant to these compounds exhibit decreased retention of drugs over time (2). The MDR phenotype (3) is the result of the overexpression of the *mdr1* gene product at least in part, a 170–180-kD glycoprotein known as P-glycoprotein. A "drug pump" model has been proposed in which the P-glycoprotein serves as an active transporter that pumps drugs out of the cell (1, 3, 4).

The cytotoxic abilities of mAbs M195 (anti-CD33) (5) and M31 (anti-CD15) against HL60 myeloid leukemia cells and a MDR HL60 variant that overexpresses the MDR protein (RV^+ [6]), were examined. In this paper, we describe the relative resistance of RV^+ cells to specific mAbs that react against cell surface protein or carbohydrate targets, and mediate cytotoxicity via complement fixation. MDR reversal studies using verapamil and with specific mAbs to P-glycoprotein showed that a significant portion of this newly described immunological resistance is mediated by P-glycoprotein overexpression. To test whether these observations were not partly due to the selection of the RV^+ cells on vincristine or a spurious phenomenon for a single cell line, K562 cells and MDR K562/huMDR1 infectants (7), which were never selected on drugs, were analyzed in similar experiments. Taken together, the results are consistent with MDR protein overexpression mediating resistance to complement mediated cytotoxicity.

Materials and Methods

Cells and Antibodies. HL60 cells (acute myeloid leukemia, $CD15^+$, and $CD33^+$), K562 cells (chronic myeloid leukemia) and K562 clone 14 and K562 clone 18, both infected with the MDR1 gene (Mechetner, E.B., unpublished results), were maintained at Memorial Sloan-Kettering Cancer Center. RV^+ (a MDR-overexpressing HL60 variant) was the generous gift of Dr. Melvin Center (Kansas State University, Manhattan, KS) and was provided by Dr. Ellen Berman (Memorial Hospital). RV^+ cells were grown in

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the presence of 120 μM (0.1 mg/ml) of vincristine (Eli Lilly, Indianapolis, IN) to maintain the MDR phenotype.

M195 and HuM195 (anti-CD33) were prepared as described (5, 8). HuG1 Fd79 (a human IgG2 control for HuM195) (8), was the generous gift of Man Sung Co (Protein Design Labs, Mountain View, CA). M31, a murine anti-CD15 antibody was established at Memorial Sloan-Kettering Cancer Center (Scheinberg, D.A., unpublished results). T9 (anti-CD71) was purchased from Coulter Corp. (Hialeah, FL). HYB-241 was a generous gift of Dr. James O'Brien (Memorial Sloan-Kettering Cancer Center) who obtained the antibody from Dr. Lana Rittmann-Grauer (Hybritech Inc.) (9). MRK-16, a IgG2a antibody, was purchased from Kamiya Biochemical Company (Thousand Oaks, CA), and UIC2, also an IgG2a antibody (10), was provided by Ingenex, Inc.

Complement-mediated Cytotoxicity. 25 μl of cell suspension (10^6 cells/ml) was incubated with 25 μl of diluted rabbit baby complement (Pel-Freeze Biologicals, Rogers, AK) and 25 μl of a serial dilution of mAbs (M31, M195, or T9) at 37°C and 5% CO_2. Cell kill was assayed using trypan blue at 30 or 60 min for M31 and M195, respectively. For cell kill using the T9 antibody, the incubation times were 60 and 120 min. Reversal of complement-mediated cytotoxicity by verapamil for RV^+ cells was assayed as above except that from a 100-mM stock solution of verapamil, either a final concentration of 5-μM or a 10-μM solution of verapamil was added at the same time as the antibody.

For the reversal of complement-mediated cytotoxicity by HYB-241 or UIC2 $F(ab)'_2$, cells were washed and resuspended to a concentration of 10^6 cells/ml. UIC2 was cleaved with pepsin to yield electrophoretically pure $F(ab)'_2$ fragments. HYB-241 antibody or UIC2 $F(ab)'_2$ was added at a final concentration of 20 μg/ml to 25 μl of cells. HYB-241 antibody was incubated with cells at 25°C for 60 min, whereas UIC2 fragment was incubated with the cells at 4°C for 60–90 min. The remaining steps were the same as described above.

Antibody-dependent Cellular Cytotoxicity. 5-h chromium release assays were conducted as described (8) using PBMC from human volunteers as effector cells and HL60 and RV^+ cells as positive targets.

Daunorubicin Resistance and Reversal of Daunorubicin Resistance with MRK-16 or UIC2 Monoclonal Antibodies. HL60 and RV^+ cells were washed and resuspended in complete RPMI media to a concentration of 5×10^5 cells/ml. 0.1 ml of cells and 0.1 ml of daunorubicin (Chiron Therapeutics, Emeryville, CA) were then added. The mixture was incubated for 48 h at 37°C in an atmosphere of 5% CO_2. Cell kill was assayed by trypan blue exclusion.

For reversal, MRK-16 or UIC2 was added to the cells at a final concentration of 5 or 20 μg/ml, respectively, and the mixture was incubated for 60 or 90 min at 4°C. Varied amounts of daunorubicin were then added. This mixture was then incubated for 24 or 48 h at 37°C and 5% CO_2, before MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyltetrazolium bromide) assay.

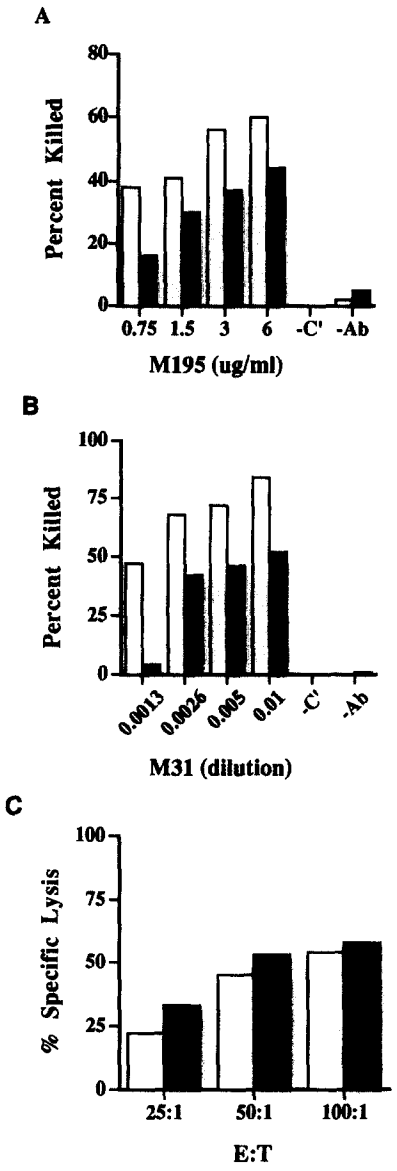

Figure 1. Relative resistance of MDR protein expressing RV^+ cells (*black*) versus HL60 (*open*) to complement-mediated cytotoxicity. (*A*) Resistance to M195 (an IgG, anti-CD33 antibody) plus complement. (*B*) Resistance to M31 (an IgM, anti-CD15 antibody) plus complement. Cell kill was assayed using trypan blue at 30 or 60 min for M31 and M195, respectively. Controls included: no complement added (*−C'*); no antibody added (*−Ab*). When neither antibody nor complement was added, no killing was seen (not shown). The data shown are a mean of duplicate measurements; however, the figure is a representative of eight experiments with similar results. (*C*) Sensitivity of RV^+ cells to specific cell lysis by M195 ADCC. Percent specific lysis by M195 and human peripheral blood effectors (*E*) and HL60 (*open*) and RV^+ (*black*) targets (*T*) determined by chromium release assays. Assays were conducted in quadruplicate as described (14). Controls without antibody or effectors were negative.

Results and Discussion

We confirmed that the RV^+ cells were relatively resistant to daunorubicin (ED_{50} = 125 ng/ml or 240 pM) in comparison with HL60 cells (ED_{50} = 4 ng/ml or 8 pM). In contrast, RV^+ cells were more sensitive to cytosine arabinoside than HL60 cells (data not shown). MDR cells in general do not exhibit cross-resistance to cytosine arabinoside. There was significant expression of P-glycoprotein on the surface of RV^+ cells (mean peak fluorescence = 3.526) but not on HL60 (mean peak fluorescence = 0.315) cells as shown by flow cytometry, using indirect immunofluorescence with MRK-16, an antibody specific for P-glycoprotein (11). It was possible to partially reverse drug resistance to daunorubicin in this HL60/RV^+ system by use of spe-

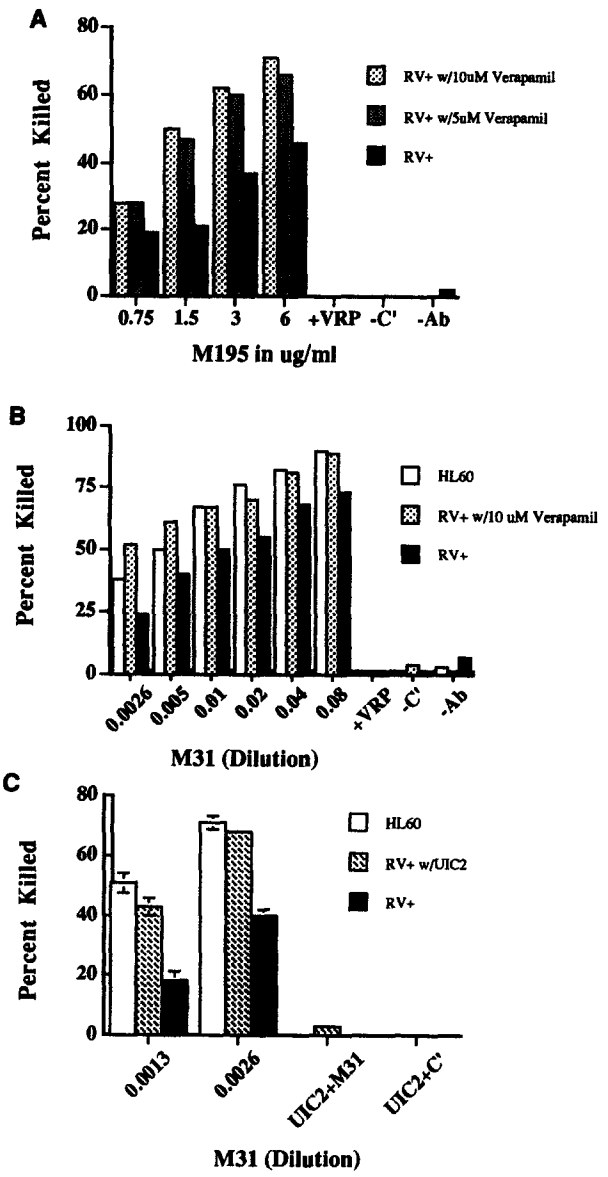

Figure 2. Reversal of resistance of RV^+ cells to complement-mediated cytotoxicity. (*A*) Reversal of resistance of RV^+ cells to complement-mediated cytotoxicity by addition of verapamil to a final concentration of 5- or 10-μM solution, using HuM195 antibody. Verapamil alone at a final concentration of 10 μM (*+VRP*) had no cytotoxic effects. Controls without complement (*−C'*) or without antibody (*−Ab*) were negative. (*B*) Reversal of resistance of RV^+ cells to complement-mediated cytotoxicity using M31 antibody by addition of a 10-μM solution of verapamil. Controls are as described in *A*. The data are a mean of duplicate measurements and the figure is a representative of four experiments. (*C*) Reversal of resistance of RV^+ cells to complement-mediated cytotoxicity using the anti–P-glycoprotein mAb, UIC2. UIC2 $F(ab)'_2$ was added to 25 μl of cells at a final concentration of 20 μg/ml, and was incubated with the cells at 4°C for 60 min. The data shown are mean ± standard deviation of experiments performed in quadruplicate. Samples that have no error bars have stardard deviations that are too small for representation on the graph. *C* is representative of three experiments.

cific anti-P-glycoprotein antibodies, as previously described (10, 12). Both UIC2 and MRK-16 antibodies were able to reverse by ~50% the daunorubicin resistance exhibited by RV^+ cell at a wide range (1–125 ng/ml) of daunorubicin concentrations (data not shown). Thus, the RV^+ cells displayed a P-glycoprotein–mediated resistance to daunorubicin (12, 13).

The levels of CD33 and CD15 target sites on HL60 and RV^+ were measured to insure that possible differences between the cells were not simply an artifact of different antigen density of the RV^+ cells. Quantitative indirect flow cytometry (5) showed that RV^+ cells had a density of CD15 and CD33 antigen expression approximately twofold greater than the parental HL60 cells (data not shown).

In the presence of complement, M195 was able to kill the HL60 cells more potently and more effectively than the RV^+ cells over a wide range of antibody concentrations (Fig. 1 *A*). M195 reacts with a protein antigen (gp67) expressed at a density of about 10,000 sites per cell, and complement-mediated killing is reduced by internalization of the antibody–antigen complex (5, 8). RV^+ cells were also more resistant than HL60 cells to a wide range of effective concentrations of an IgM, M31, that recognizes the carbohydrate epitope Lewis X (CD15) (Fig. 1 *B*). Therefore, the immunological resistance is restricted neither to protein targets nor to IgG-mediated cytotoxicity.

In contrast, as had been shown previously (14), RV^+ cells were not resistant to antibody-dependent cellular cytotoxity (ADCC) (Fig. 1 *C*) or to LAK-mediated killing. Interestingly, ADCC may work through two distinct mechanisms, perforin-mediated lysis and Fas-ligand–mediated apoptosis (15). The dual mechanism of killing could contribute to this lack of resistance.

Complement-mediated cytotoxicity experiments were performed in the presence of verapamil, a potent inhibitor of the ability of the P-glycoprotein to confer the MDR phenotype (16, 17). The addition of verapamil to the complement cytotoxicity assays abrogated the resistance of RV^+ cells and resulted in more efficient killing (Fig. 2, *A* and *B*) comparable to that for HL60 cells. Reversal of the resistance phenotype was obtained at several concentrations of verapamil ranging from 5 to 10 μM. Controls performed with cells and verapamil without antibody or complement showed that the increased cytotoxicity was not due to verapamil itself which may be toxic over far longer periods (days, not minutes).

P-glycoprotein–specific mAbs were used to confirm that the MDR reversal observed with verapamil was the result of P-glycoprotein inhibition. As described above, anti–P-glycoprotein antibodies, UIC2 and MRK-16, can partially reverse the daunorubicin resistance, as can HYB-241 (9). Both UIC2 and MRK-16, but not HYB-241, direct complement-mediated cytotoxicity themselves on cells expressing P-glycoprotein (data not shown); therefore an $F(ab)'_2$ was made by pepsin cleavage of UIC2. Both the UIC2 $F(ab)'_2$ (Fig. 2 *C*) and HYB-241 antibodies (not shown) reversed the P-glycoprotein phenotype of RV^+ cells in assays of complement-mediated cytotoxicity by M31. The reversal compares favorably to the reversal of daunorubicin cytotoxicity. This reversal effect was seen at a wide range of other concentrations of M31 ranging from a dilution of 1/12 to 1/1,536 (data not shown). Control experiments with UIC2 $F(ab)'_2$ added to HL60 plus M31 and complement

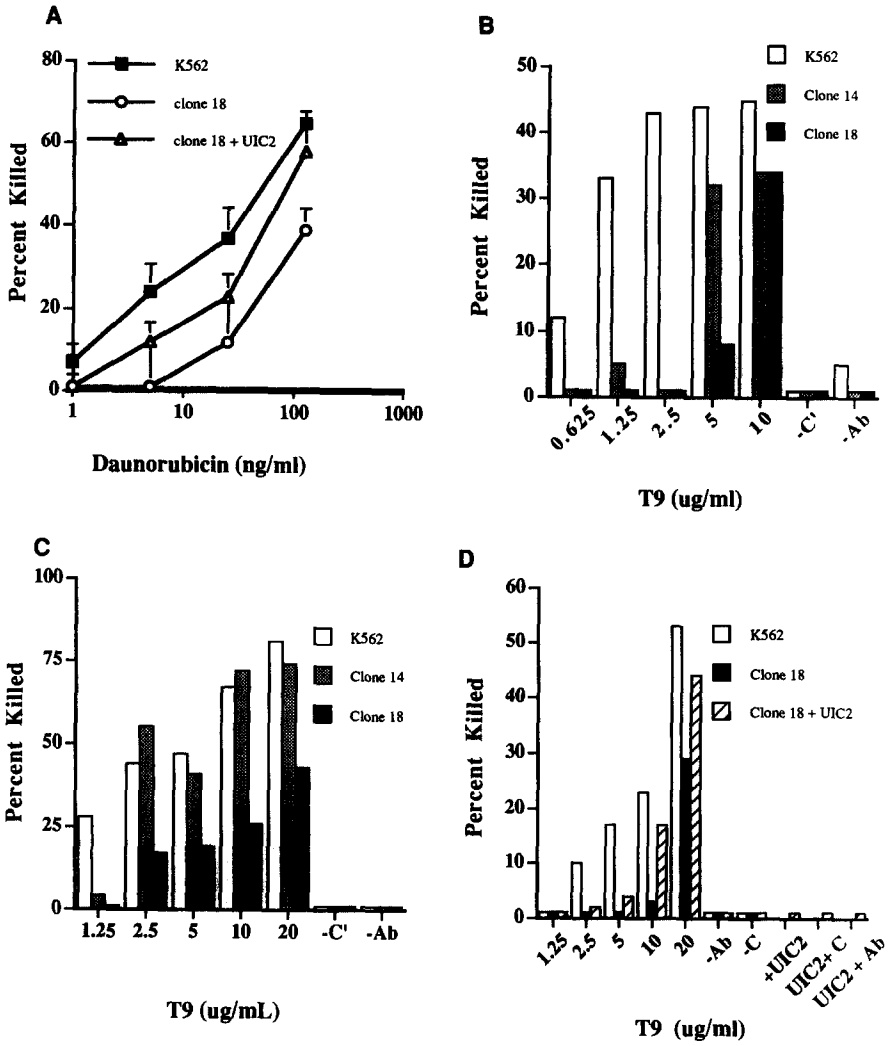

Figure 3. Resistance and UIC2-mediated reversal of resistance of clone 14 and clone 18 cells to daunorubicin and complement-mediated killing. Trypan blue exclusion was performed to determine cell viability at 48 h. (*A*) K562 and clone 18 cells treated with daunorubicin and UIC2. K562 cells (*squares*); clone 18 (*circles*); clone 18 with UIC2 (*triangles*). The data shown are mean values of experiments performed in quadruplicate. Samples that have no error bars have standard deviations that are too small for representation on the graph. UIC2 alone had no effect on cell viability for either cell line. (*B*) Addition of T9 plus complement for 60 min. (*C*) Addition of T9 plus complement for 120 min. Cell kill was determined by trypan blue exclusion. No killing was seen in controls in which no complement was added (−*C*); no antibody was added (−*Ab*); and neither antibody nor complement was added. The data shown are a mean of duplicate measurements, and therefore standard deviation is not shown. (*D*) Reversal of resistance to T9 plus complement for 120 min with UIC2. UIC2 antibody was added to 25 μl of cells at a final concentration of 20 μg/ml. UIC2 alone, or with T9 or complement, was not toxic. The data shown are a mean of duplicate measurements.

did not increase complement-mediated cytotoxicity (data not shown), thus strengthening the relationship between P-glycoprotein and complement resistance. Controls with UIC2 and verapamil alone were not cytotoxic to the cells.

To confirm that these results were not an isolated phenomenon of the HL60/RV$^+$ system or a consequence of the selection of the RV$^+$ cells on vincristine, K562 cells that were infected with the MDR1 gene also were examined for resistance to daunorubicin and complement-mediated cytotoxicity. As determined by flow cytometry, clone 14 and clone 18 cells, like RV$^+$ cells, had significant expression of P-glycoprotein on their surface, whereas the parental K562 cells had none (data not shown). Clone 18 cells expressed more P-glycoprotein than clone 14 cells, characterized by densitometry of protein concentrations on a Western blot using anti–P-glycoprotein antibody and by flow cytometry (data not shown).

Clone 14 and clone 18 cells were significantly resistant to daunorubicin cytotoxicity (LD_{50} of 125 and >125 ng/ml, respectively) as compared with the sensitive K562 cells (LD_{50} of 15 ng/ml). Resistance was directly related to P-glycoprotein expression as this resistance was partially reversible with the addition of the mAb UIC2 to P-glycoprotein (Fig. 3 *A*).

K562 cells do not express significant amounts of CD33 or CD15. Therefore, an IgM mAb to CD71 (T9) was used instead. Quantitative flow cytometry for levels of CD71 on the K562 cells and the MDR-positive clones showed that the resistant clones have a slightly higher density of CD71 than the K562 cells (data not shown). Clones 14 and 18 were resistant to complement-mediated cytotoxicity (Fig. 3 *B*). The resistance of clone 14 to complement-mediated cytotoxicity could be overcome by increasing the length of exposure to complement from 60 to 120 min, whereas clone 18 cells remained resistant (Fig. 3 *C*). Clone 18 is more resistant to daunorubicin than clone 14 and was more resistant to complement-mediated cytotoxicity. The direct relationship between P-glycoprotein expression on the three K562 cell lines and both their drug and complement resistance strengthens the association between P-glycoprotein function and resistance to complement-mediated cytotoxicity.

UIC2 F(ab)$'_2$ was then used to reverse the resistance of clone 18 to complement-mediated cytotoxicity. As was observed in the HL60/RV$^+$ system, UIC2 F(ab)$'_2$ was able to

partially reverse the MDR phenotype (Fig. 3 *D*). Although the sensitivity to complement is not completely restored, the reversal does compare well to the reversal of daunorubicin cytotoxicity (Fig. 3 *A*). UIC2 F(ab)'$_2$ alone was found not to be cytotoxic to the cells.

One of the assumptions of an immunological approach to the treatment of cancer is that cells that are resistant to cytotoxic drugs because of prior exposure should not be cross-resistant to immunotherapy. Hence, the cross-resistance observed here was surprising. Indeed, ADCC via specific antigen recognition was increased on RV$^+$ cells, as expected, as the RV$^+$ cells express increased target antigen (14). In addition to their sensitivity to ADCC, the RV$^+$ cells were killed more effectively than the HL60 cells in cytotoxicity experiments with cytosine arabinoside, a drug to which MDR tumor cells do not develop cross-resistance. RV$^+$ cells were also more sensitive to LAK cells, another relatively nonspecific agent.

The theory that P-glycoprotein acts as an active efflux pump for drugs (18) fails to explain the resistance to complement-mediated cytotoxicity with three different antibodies, an IgG and two IgM. If resistance to the complement is mediated by MDR, then a suitable explanation of its mechanism must take into account the effects of agents acting on the outside of the cell.

By the use of verapamil and three different specific antibodies, we were able to confirm that P-glycoprotein itself was involved in the resistance to complement-mediated cytotoxicity in two different systems. The observation that UIC2 F(ab)'$_2$ did not increase complement-mediated cytotoxicity when in the presence of HL60 cells plus complement, strongly reaffirms P-glycoprotein's involvement with complement resistance. In addition, both drug and complement resistance in this system correlated directly with the levels of P-glycoprotein expression. However, because the RV$^+$ cells were derived by selection in vincristine, it is possible that other mechanisms of drug resistance are present in these cells, but not in the K562 system.

Other explanations for the broad resistance observed might include altered binding of the antibodies to their target or a change in the internalization kinetics of the conjugates, yielding decreased residence time at the surface. The kinetics of internalization of M195 bound to RV$^+$ and HL60 cells are similar, however (data not shown). The increased sensitivity to ADCC argues against a significant decrease in antigen binding, since decreased binding would conversely reduce ADCC. Thus, alterations in antigen binding or internalization rates fail to provide a comprehensive explanation for the resistance we observe.

Significant changes in plasma membrane electrochemical potential have been observed in MDR cells (19) and these appear to be due to P-glycoprotein (20). These changes typically include elevated intracellular pH (pH_i), which is also referred to as increased ΔpH, as well as decreased (less negative) electrical membrane potential ($\Delta\Psi$). These changes likely have broad effects on many events that occur at the cell surface. Compared with HL60 cells, RV$^+$ cells have a significant elevated pH_i as well as a decreased $\Delta\Psi$ (Weisburg, J.H., D.A. Scheinberg, and P.D. Roepe, unpublished results); thus, it is possible that these changes contribute to the resistance we measured. Indeed the pore-forming protein from *Trypanosoma cruzi*, which is immunologically related to human C9 protein (the ninth component of complement) forms "pores" (or "complement channels") readily at lower pH but very inefficiently at higher pH (21). In addition, Jackson et al. (22) have reported rather significant decreases in mean complement channel conductance, as well as altered efficiency of channel formation, for muscle cell membranes clamped to -47 versus -60 mV.

The relationship between the MDR phenotype and complement resistance raises questions about the physiologic role of P-glycoprotein in protection against autolysis. Interestingly, CD34-positive hematopoietic stem cells express P-glycoprotein (23) as do many normal human tissues (24). IL-2 transport may also be modulated by P-glycoprotein (25). It is hypothesized that the function of P-glycoprotein is to protect against cytotoxic plant alkaloids and other dietary xenobiotic substances (24). Many of the sites where P-glycoprotein is found normally in the body are also sites of bacterial entry and inflammation. It could be hypothesized that P-glycoprotein may have evolved to protect the body against endogenous toxins, such as complement, instead of, or in addition to, exogenous substances. Additional work to elucidate the mechanism of resistance and kinetics of pore formation in the MDR cells is in progress.

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