Intracellular pH and Multidrug Resistance Regulate Complement-mediated Cytotoxicity of Nucleated Human Cells*

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In previous work (Weisburg, J. H., Curcio, M., Caron, P. C., Raghi, G., Mechetner, E. B., Roepe, P. D., and Scheinberg, D. A. (1996) J. Exp. Med. 183, 2699–2704), we showed that multidrug resistance (MDR) cells created by continuous selection with the vinca alkaloid vincristine (HL60 RV+) or by retroviral infection (K562/human MDR 1 cells) exhibited significant resistance to complement-mediated cytotoxicity (CMC). This resistance was due to the presence of overexpressed P-glycoprotein (P-GP). In this paper, we probe the molecular mechanism of this phenomenon. We test whether the significant elevated intracellular pH (pHi) that accompanies P-GP overexpression is sufficient to confer resistance to CMC and whether this resistance is related to effects on complement function in the cell membrane. Control HL60 cells not expressing P-GP, but comparably elevated in cytosolic pHi by two independent methods (CO2 “conditioning” or isotonic Cl− substitution), are tested for CMC using two different antibody-antigen systems (human IgG and murine IgM; protein and carbohydrate) and two complement sources (rabbit and human). Elevation of pHi by either of these methods or by expression of P-GP confers resistance to CMC. Resistance is not observed when the alkalization mediated by reverse Cl−/HCO3− exchange upon Cl− substitution is blocked by treatment with dihydro-4,4′-disothiocyanostilbene-2,2′-disulfonate. Continuous photometric monitoring of 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF), to assess changes in pHi or efflux of the probe through MAC pores, in single cells or cell populations, respectively, verifies changes in pHi upon CO2 conditioning and Cl− substitution and release of BCECF upon formation of MAC pores. Antibody binding and internalization kinetics are similar in both the parental and resistant cell lines as measured by radioimmunoassay, but flow cytometric data showed that net complement deposition in the cell membrane is both delayed and reduced in magnitude in the MDR cells and in the cells with increased pHi. This interpretation is supported by comparison of BCECF release data for the different cells. Dual isotopic labeling of key complement components shows no significant change in molecular stoichiometry of the MACs formed at different pHi. The results are relevant to understanding clinical implications of MDR, the physiology of P-GP, and the biochemistry of the complement cascade and further suggest that the “drug pump” model of P-GP action cannot account for all of its effects.

A tumor multidrug resistance (MDR)1 phenotype is frequently (but not always) due to the overexpression of the mdr1 gene product, a 170–180-kDa glycoprotein known as P-glycoprotein (P-GP) or human MDR 1 protein (1). A careful distinction should be made between the MDR phenotype(s) observed in cells exposed to or selected with various chemotherapeutic drugs versus the phenotype mediated solely by P-GP overexpression (2). One hypothesis proposed to explain the contribution of P-GP to MDR is the “drug pump” model, in which P-GP hydrolyzes ATP to actively efflux a variety of chemotherapeutic drugs such as anthracyclines, vinca alkaloids, and epipodophyllotoxins out of the cell against a concentration gradient (1, 3). Although this model accounts for some of the properties of MDR cells, it fails to explain resistance to complement-mediated cytotoxicity (CMC) observed in some MDR cells (4) and other phenomena (reviewed in Ref. 5).

Many investigators working to improve cancer therapy have proposed that immunologic approaches to killing cancer cells (such as CMC) might circumvent chemotherapeutic drug resistance. One of the assumptions of an immunological approach to the treatment of cancer is that cells that are resistant to cytotoxic drugs (e.g. MDR tumor cells) should not be cross-resistant to immunotherapy, since the mechanisms of cytotoxicity are so widely different. Because complement mediates cytotoxicity via mechanisms directed to the outside surface of the cell, the protective functions of P-GP overexpression in MDR tumor cells should not be expected to be relevant to conferring resistance to CMC; i.e., antibody molecule juxtapositioning following binding to specific antigen initiates the complement cascade, culminating in the formation of the membrane attack complex (MAC). When MACs are inserted into the plasma membrane of the cell, nonspecific pores as large as 100 Å in diameter are formed, causing cell lysis (6). We showed previously, however, that a MDR HL60 variant (HL60 RV+), selected on vincristine that also overexpresses P-GP is resistant to CMC relative to the parental HL60 myeloid leukemia cells. Differences in killing were independent of antibody isotype (either IgG and IgM) and target antigen. Resistance to CMC was also observed in K562 cells overexpressing P-GP via retroviral infection and not preselected with chemotherapeutic drugs. The immunological resistance in both MDR cell lines

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1 The abbreviations used are: MDR, multidrug resistance; pHi, intracellular pH; CMC, complement-mediated cytotoxicity; P-GP, P-glycoprotein; MAC, membrane attack complex; Vmem, membrane potential; BCECF-AM, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein; AM, ace-toxymethyl ester; HBSS, Hanks’ balanced salt solution; PBS, phosphate-buffered saline; H2DIDS, dihydro-4,4′-disothiocyanostilbene-2,2′-disulfonate.
was reversed by the calcium channel blocker verapamil and the F(ab\(^\prime\))\(_2\) fragments of the P-GP-inactivating monoclonal antibody UIC2. These data are consistent with P-GP overexpression mediating resistance to CMC. Elucidating the molecular mechanism of this resistance phenomenon is important, because frequently invoked models for how P-GP contributes to MDR (e.g. the “drug pump” models) do not provide a framework for explaining P-GP-mediated resistance to CMC.

MDR cells typically have elevated intracellular pH (pH\(_i\)) and decreased plasma membrane potential (V\(_m\)) (7–12), and this is apparently due to P-GP overexpression and not exposure to chemotherapeutic drugs (2). Hence, another model for P-GP’s action (frequently referred to as the altered partitioning model) suggests that alterations in pH\(_i\) and/or V\(_m\) that accompany the overexpression of P-GP indirectly affect partitioning of chemotherapeutic drugs, but P-GP does not directly pump them (5, 9, 11, 12). This model may offer a more reasonable framework for addressing resistance to CMC in MDR cells.

Higgins, Sepulveda, and colleagues (13–15) have suggested that P-GP may translocate Cl\(^-\) directly or indirectly. Cytosolic Cl\(^-\) concentration and Cl\(^-\) plasma membrane permeability are generally important in maintaining pH\(_i\) and V\(_m\) for eukaryotic cells. Altered Cl\(^-\) permeability and altered pH in MDR cells may indeed be connected in some fashion, since recent Cl\(^-\) substitution experiments revealed unusual and extensive pH\(_i\) changes in human MDR 1 transfected (16). Overexpression of P-GP inhibits Cl\(^-\) and HCO\(_3\)^- dependent pH\(_i\) homeostasis, and this is apparently due to specific inhibition of (or competition against) Na\(^+\)-independent Cl\(^-\)/HCO\(_3\)\(^-\) exchange (anion exchange (10, 16, 17)). HL60 cells are known to possess Cl\(^-\)/HCO\(_3\)\(^-\) exchange activity (18), so P-GP expression in these cells would be expected to perturb pH\(_i\).

Thus, in this study we examined resistance in the HL60/RV\(^+\) system and its relationship to pH\(_i\) perturbations. Two independent approaches (Cl\(^-\)/glucionate exchange and CO\(_2\) conditioning) were used to alkalize control HL60 cells not expressing P-GP. Intracellular alkalization of HL60 cells conferred CMC resistance similar to that measured for the RV\(^+\) cells expressing P-GP. Resistance did not develop when intracellular alkalization upon Cl\(^-\)/glucionate exchange was prevented via inhibition of the Cl\(^-\)/HCO\(_3\)\(^-\) exchanger due to the addition of dihydro-4,4\'-disothiocyanostilbene-2,2\'-disulfonate (H\(_2\)DIDS). Furthermore, the pH\(_i\) changes did not appear to change antibody binding and modulation kinetics but did appear to have effects on complement membrane attack complex (MAC) deposition.

The final step of the complement cascade is formation of MACs, which are large pores in the plasma membrane of the cell that destroy osmotic balance and lead to cell lysis. MACs consist of five interacting proteins (C5b, C6, C7, C8, and C9) that, when sequentially activated upon antibody binding to the target cell, expose hydrophobic residues and insert into the membrane. Association of these five proteins within the membrane leads to formation of nonspecific trans-membranous channels (19, 20). “Sublytic” dilute concentrations of complement proteins may form precursors of the full MAC (e.g. “partial MACs”) that are more specific with regard to transport properties and perhaps even voltage-regulated (21). The pore diameter of the completely assembled MAC is variable and is essentially determined by the number of C9 molecules that contribute to pore formation. MAC stoichiometry is described as (C5b,C6,C7,C8)\(_n\) C9\(_n\), where n = 9–18.

We measured the formation of functional pores with different characteristics by continuous monitoring of fluorescence methods (9) and determined the molecular stoichiometry of MAC structure by purification of MAC complexes harboring radiolabeled subunits. The data suggest that increased pH\(_i\) reduces the rate of formation of functional “full MACs” (i.e. (C5b-C8), (C9)) in the cell membrane of target cells.

The results have important consequences for better understanding the role of P-GP overexpression and chemotherapeutic drug exposure in tumor resistance phenomena. They also define an important biochemical parameter for efficient manipulation of immune mediated killing and the study of the terminal complement cascade.

**EXPERIMENTAL PROCEDURES**

**Materials—**2,7'-Bis(carboxyethyl)-5,6-carboxyfluorescein acetoxyethyl ester (BCECF-AM) and nigericin were purchased from Molecular Probes, Inc. (Eugene, OR; H\(_2\)DIDS was purchased from Sigma; and baby rabbit complement was purchased from Pel Freez (Brown Deer, WI). All were used without additional purification. Cell-Tak was from Becton Dickinson and was used as described by the manufacturer. Human complement (plasma) was obtained from normal donors.

**Cells and Antibodies—**HL60 cells (acute myeloid leukemia, CD15\(^+\), and CD33\(^+\)) were maintained at Memorial Sloan-Kettering Cancer Center. RV\(^+\) (a P-GP expressing MDR 1 variant selected by continuous exposure to vincristine) was the generous gift of Dr. Ellin Berman (Memorial Hospital). RV\(^+\) cells were grown in RPMI plus 10% fetal calf serum at 37 °C in an atmosphere of 5% CO\(_2\) and in the presence of 120 mM (0.1 mg/ml) vincristine (Lilly) to maintain the MDR phenotype. These cells were cultured without vincristine for one passage prior to experiments. This practice did not reduce MDR or the level of P-GP expression.

The humanized monoclonal antibody M195 was prepared as described (22, 23), and M31, a murine anti-CD15 antibody, was established at Memorial Sloan-Kettering Cancer Center.\(^\text{2}\) Rabbit anti-human C9 anti-C5b-9 antibody was purchased from Calbiochem (La Jolla, CA, U.S.A.).

**Single Cell Photometry and Manipulation of pH\(_i\)—**As described previously (10, 16, 24), we have constructed a single cell photometry apparatus using a Nikon diaphot epifluorescence microscope and a Photon Technology alpha-scan fluorometer. For BCECF experiments, a 510-nm dichroic and 530-nm bandpass filter was positioned beneath the stage, and the excitation monochromator was flipped between 439 and 499 nm by the computer (see Ref. 24 and references therein for additional detail).

Cells (1.0 × 10\(^3\)) were washed and resuspended in 200 μl of a solution consisting of 50 μl RPMI 1640 and 150 μl sterile filtered HBSS (118 mM NaCl, 5 mM KCl, 24.2 mM NaHCO\(_3\), 1.3 mM CaCl\(_2\), 0.5 mM MgCl\(_2\), 0.6 mM NaHPO\(_4\), 0.5 mM KHPO\(_4\), 10 mM glucose). The cells were incubated with 5 μM BCECF-AM for 20 min at 37 °C and 5% CO\(_2\). Next, the cells were adhered to glass coverslips (Corning Glassworks, 18 mm\(^2\), 0.11-mm thick) using Cell-Tak (Becton Dickenson) and incubated for an additional 20 min at 37 °C and 5% CO\(_2\) in the continued presence of BCECF-AM. Coverslips were then mounted in a home-built perfusion chamber (10) and continuously perfused at a constant rate (approximately 5 ml/min) with HBSS buffer equilibrated with 5% CO\(_2\) and to 37 °C. Uniform BCECF staining was verified visually and by monitoring the intensity of 490 nm excitation (535 emission) and was found to be similar between the cell lines. Excitation was limited to the time of data collection to limit photobleaching. HBSS was continuously purged with 5% CO\(_2\) and a fine jet of 5% CO\(_2\) was directed over the mounted coverslip to maintain buffer pH\(_i\), which was monitored with a microelectrode. Calibration of intracellular BCECF was by the K\(^+\)/nigericin titration approach (25), but in single cell mode (10, 16, 24), wherein HBSS harboring nigericin was continuously flowed over the cells. Steady state pH\(_i\) was calculated using the best fit to the calibration data (described in detail in Ref. 2).

To elevate pH\(_i\) of the control HL60 cells, which do not express measurable P-GP (8), two approaches were used. First, HL60 cells underwent elevation of pH\(_i\) by manipulation of external CO\(_2\) as described (2). In brief, cells were grown at 5% CO\(_2\) in normal RPMI 1640; shifted to a 10% CO\(_2\) atmosphere, which causes rapid acidification of the medium; and then grown in this atmosphere for 2–7 days. The “acid-conditioned” cells were then placed back in a 5% CO\(_2\) atmosphere in the presence of fresh pre-equilibrated (to 5% CO\(_2\)) medium for 1 or 2 h.

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\(^\text{2}\) D. A. Scheinberg and M. Tanimoto, unpublished results.
As described (Ref. 2; see "Results") this results in an "overshoot" of pH, since alkalizing mechanisms induced by growth at 10% CO2 are still functioning (at least for several hours) in the reduced CO2 environment. Detailed analysis of the conditioning method is presented in Ref. 2.

Alternatively, pH was elevated by Cl⁻ substitution as described (10, 16). In these experiments, HBSS per se was rapidly replaced with Cl⁻-free HBSS (glucuronate isotonically replacing Cl⁻). Control experiments (not shown) showed that excess glutamate tended to inhibit the complement cascade (see Ref. 26), so glutamate was preferred in isosmotic Cl⁻ substitution. Long term Cl⁻ substitution experiments performed essentially as described (16) verified that HL60 cells were then washed free of medium, resuspended in HBSS plus H2DIDS (3.20 mM) and pH 7.30 (by perfusion with 5% CO2). The cells were allowed to equilibrate in HBSS containing 5% fetal calf serum for 10 min and then plated for the cytotoxicity measurements (4). For the Cl⁻-free HBSS experiments, the cells were pretreated with HBSS as above, washed with Cl⁻-free HBSS to remove Cl⁻, and then resuspended in Cl⁻-free HBSS at 37 °C, pH 7.30, with 5% fetal calf serum. Antibody and complement dilutions were made in either HBSS or Cl⁻-free HBSS as appropriate.

To examine whether anion exchange was responsible for the alkalization of the cells under these Cl⁻ substitution conditions, H2DIDS, a well known anion exchange inhibitor, was added to the cells at various concentrations (250 μM to 1 mM) after washing with BCECF. The cells were incubated with the H2DIDS for 20 min at 37 °C, and isosmotic Cl⁻/glucuronate exchange was performed as above in the presence of CO2/HCO3⁻ to examine any inhibition of Cl⁻/HCO3⁻ exchange (see "Results").

**Complement-mediated Cytotoxicity in HBSS or Cl⁻-free HBSS**—Complement-mediated cytotoxicity assays were conducted essentially as described (4) with glutamate rather than Cl⁻. At 5, 10, 15, 20, and 30 min of incubation, a 1:10 dilution of M31 antibody and 800 μl of undiluted human washed control MCMs were added to the cells and photometrically monitored for trypan blue exclusion.

**Radiolabeling Complement Components C7 and C9**—Proteins were iodinated using the chloramine-T method and purified by size exclusion chromatography (27). C9 was labeled with 125I; C7 was labeled with 131I.

**Determination of Pure Size and Number of MACs on HL60 Cells under Cl⁻-free Conditions**—We modified the protocol previously published by Ware and colleagues (28). 5.0 x 10⁶ HL60 cells were washed three times with HBSS, pH 7.30, to remove all growth media. Next, the cells were resuspended in HBSS plus 10% fetal bovine serum and incubated for 10 min at 37 °C. For Cl⁻-free conditions, the HL60 cells were then washed with two 1:1 Cl⁻-free HBSS (equimolar glucuronate and chloride) solutions prior to the 10 min wash with HBSS at 37 °C to remove all medium and resuspended in HBSS pre-equilibrated to 37 °C and pH 7.30 (by perfusion with 5% CO2). The cells were then allowed to equilibrate in HBSS containing 5% fetal calf serum for 10 min and then plated for the cytotoxicity measurements (4). For the Cl⁻-free HBSS experiments, the cells were pretreated with HBSS as above, washed with Cl⁻-free HBSS to remove Cl⁻, and then resuspended in Cl⁻-free HBSS at 37 °C, pH 7.30, with 5% fetal calf serum. Antibody and complement dilutions were made in either HBSS or Cl⁻-free HBSS as appropriate.

To examine if Cl⁻/HCO3⁻ exchanger function was involved in any resistance to complement when the cells were suspended in Cl⁻-free HBSS, cells were treated with H2DIDS prior to the CMC assay. HL60 cells were washed free of medium, resuspended in HBSS plus H2DIDS (concentrations ranged from 250 μM to 1 mM), and then incubated for 20 min at 37 °C in an atmosphere of 5% CO2. The cells were washed with Cl⁻-free HBSS and then plated for cytotoxicity with an amount of antibody and complement known from previous experiments to yield approximately 50% killing (see "Results").

**Radiolabeling of HuM195**—Proteins were iodinated using the Chloramine T method and purified by size exclusion chromatography (27).

**Modulation and Internalization of 125I-HuM195—**The retention time of free radiolabeled C7 and C9 (uncomplexed with other MAC components) was determined by gel filtration chromatography (27). The antibody binds to the neopeptide of C9 that forms during polymerization, but it does not react with free C9. Anti-C5b-9 antibody was incubated with the cells for 30 min on ice, unbound antibody was then removed by washing the cells with PBS, and goat anti-mouse-IgG-fluoroscein isothiocyanate was added and allowed to bind to primary antibody for 45 min on ice and in the dark. The cells were then washed with PBS and resuspended in 500 μl of 2% parafomaldehyde. Samples were analyzed on an EPICS-Profile II flow cytometer (Coulter, Hialeah, FL). Cell viability of the samples collected at 30 min was determined by trypan blue exclusion.

**Flow Cytometric Analysis of MAC Formation Kinetics—**1.0 x 10⁶ HL60 or RV⁻ cells were washed and resuspended with RPMI medium. The cell pellet was then resuspended in 800 μl of a 1:10 dilution of M31 antibody and 800 μl of a 1:3 dilution of human serum. In control tubes, medium was added instead of the human serum. The cells plus antibody and serum were incubated at 37 °C. At 5, 10, 15, 20, and 30 min of incubation, a 160-μl aliquot was removed, and 2 ml of stripping buffer (see above) was added. This was done to prevent the secondary antibody (goat anti-mouse fluoroscein isothiocyanate-labeled antibody) from reacting nonspecifically with the IgM (murine M31 antibody) bound to the cell surface. The cells were incubated with stripping buffer for 10 min at room temperature and then centrifuged at 750 x g and 4 °C. The cell pellet was resuspended in 500 μl of ice-cold 2% paraformaldehyde and allowed to incubate on ice for 15 min in order to fix the MACs. The paraformaldehyde was then washed by washing three times with PBS, and an anti-MAC (C5b-9)-antibody was then added to the cells. This antibody binds to the neopeptide of C9 that forms during polymerization, but it does not react with free C9. Anti-C5b-9 antibody was incubated with the cells for 30 min on ice, unbound antibody was then removed by washing the cells with PBS, and goat anti-mouse-IgG-fluoroscein isothiocyanate was added and allowed to bind to primary antibody for 45 min on ice and in the dark. The cells were then washed with PBS and resuspended in 500 μl of 2% parafomaldehyde. Samples were analyzed on an EPICS-Profile II flow cytometer (Coulter, Hialeah, FL). Cell viability of the samples collected at 30 min was determined by trypan blue exclusion.
BCECF exited the cells (due to an increase in quantum yield for solution-based BCECF versus intracellular BCECF, see “Results”). Changes in 439-nm fluorescence are not expected upon any possible movement of H⁺ during the experiment, since 439 nm is isobestic with regard to changes in pH. This was confirmed by titration of BCECF in the cuvette (not shown). Parallel experiments wherein cells and supernatant were separated and fluorescence in each fraction was quantified individually (not shown, but see Ref. 9) verified that the change in 439-nm BCECF fluorescence was indeed due to exit of the probe from the cell. As an additional control experiment, 200 µl of heat-inactivated rabbit complement was added to the cells plus M51 antibody (see “Results”) in order to test whether any change in probe signal was caused by the addition of reagents but in the absence of functional pores.

In analyzing these data, we also found that the change in quantum efficiency of 490-nm excitation is greater than the change at 439 nm upon movement of the probe from the cytosol to the extracellular space; thus, exit of the probe from cells in a continuously mixed suspension also results in a measurable change in the 439/490-nm excitation ratio (see “Results”). This is probably due to a much greater affect of solvent polarity on the transition revealed by the 490-nm peak, relative to the transition revealed at 439 nm. Such an effect complicates analysis of pH changes that might occur during an experiment wherein total BCECF response in a continuously mixed suspension of cells was being monitored. Analysis of these data to separate BCECF leak from cytosolic pH changes will be presented elsewhere.

BCECF fluorescence data were collected as PTI data files on a PTE alpha-scan fluorometer, converted to ASCII format, and imported into QuattroPro 7 software (Corel). To obtain quantitative parameters for the 439-nm BCECF, data were fit to a function of the form,

\[
F(t) = \frac{Ae^{-(t-t_s)/\sigma^2}}{(t-t_s)/\sigma^2} + C \quad (\text{Eq. 1})
\]

which is the integral of a gaussian (i.e., a sigmoid), and where \( F(t) \) is fluorescence intensity at 439 nm excitation or the 439/490-nm excitation ratio, see “Results”) as a function of time (t); and A, C, \( \sigma \), and \( t_s \) are variables. The expression under the integral has the general form of a bell-shaped (Gaussian) curve. After iterative least-squares best fit to the raw data (convergence satisfied in <100 iterations; \( \sigma < 0.05 \)), the expression perfectly fit the raw data (see “Results”). In this expression, \( C \) corresponds to the intensity of fluorescence before complement is added, \( A \) is the maximal rate of change in \( F(t) \) (e.g., \( V_{\text{max}} \), which is observed at time \( t_s \)), and \( \sigma \) defines the characteristic time of transition of \( F(t) \) to a new steady state value. We also define the “delay time” \( D \) in the equation,

\[
D = t_m - t_s \quad (\text{Eq. 2})
\]

where \( t_m \) is the time at which complement is added to the cell suspension; i.e., \( D \) is the time between the addition of complement and the maximal rate of change in \( F(t) \). These simple parameters are useful for quantifying the rate of formation of pores (i.e., the “delay time”, \( D \) as well as the cellular rate of transport (maximal rate of change, \( A \), also referred to as \( V_{\text{max}} \), which is proportional to the number of pores formed and/or the intrinsic transport properties of those pores that are formed.

RESULTS

CO₂ Conditioning Causes Alkalization of pHi in HL60 Cells—One difficulty in accurately measuring the effects of altered pHi on cellular biochemistry is the general inability to stably modulate pHi via nondeleterious means for sufficient periods of time. For example, chemotherapeutic drug resistance assays require hours to days of incubation with cytotoxic drugs, but precise manipulation of pHi via the use of ion transport inhibitors or ionophores is either toxic, transient, or both; hence, investigating the role of altered pHi in drug resistance phenomena is difficult and requires special methods (see Ref. 2). In the present study, we wished to assess the importance of pHi in CMC, which requires at least 30 min to measure appropriately; thus, we required methods to stably and safely alter pHi within this period of time. As reported previously (2), some cell populations can be manipulated to a higher average pHi by CO₂ conditioning procedures (which should be distinguished from CO₂ “pulse” methods that induce pHi changes on a much shorter time scale; see Ref. 2). Thus, upon returning HL60 cells to a 5% CO₂ atmosphere after they have been conditioned for 2–7 days in a 10% CO₂ atmosphere, the mean steady-state pHi of the cell population was alkaline (Table I). Rapidly growing HL60 cells that were constantly maintained in a 5% CO₂ incubator had average pHi of 7.27 ± 0.06. When these cells were conditioned in a 10% CO₂ environment and then placed back into medium pre-equilibrated with 5% CO₂ for 1 h, the mean pHi of the population increased by about 0.12 pH units to 7.39 ± 0.07. If the conditioned cells were resuspended in 5% CO₂ medium for 2 h, pHi increased even further to 7.53 ± 0.09, similar to the steady state pHi of RV⁺ cells of 7.52 ± 0.03 (cf. Table I). Continued incubation past this time resulted in gradual return to base-line steady state pHi (not shown, but see Ref. 2). As described previously (2), we interpret these changes in pHi to be the result of one or more alkalizing mechanisms that are up-regulated, while the cells are cultured under an increased acid burden (i.e. 10% CO₂); when the acid burden is removed suddenly (i.e. cells are returned to 5% CO₂), these alkalizing mechanisms “overshoot” pHi until such time as they are down–regulated as the cell readjusts to normal 5% CO₂. This down–regulation phase is typically not complete for several hours (see Ref. 2 for detailed description).

Cl⁻ / Glucanate Exchange Increases pHi of HL60 Cells—Upon exposure to Cl⁻–free HBSS, HL60 cells with an initial pHi near 7.25 became significantly alkaline, with pHi increasing to near 7.65 (Fig. 1A). This alkalization is dependent upon the presence of HCO₃⁻ (not shown) and is inhibited by H₂DIDS (see Fig. 1B). Previously (18), Restrepo and colleagues demonstrated the presence of a Cl⁻ / HCO₃⁻ exchanger in HL60 cells. Thus, it is likely that alkalization is mediated by “reverse” Cl⁻ / HCO₃⁻ exchange instigated by the outward directed CI⁻ flux caused by isotonic Cl⁻ substitution (see Ref. 16). Interestingly, HL60 remained stably alkaline for at least 30 min in CI⁻–free HBSS. This suggests that HL60 cells lack a compensatory Cl⁻–independent mechanism capable of restoring normal pHi under these conditions (e.g. a Na⁺ / HCO₃⁻ cotransporter). Stability of alkalization under these conditions is important, since assaying complement-mediated cytotoxicity in this system requires a 30 min incubation period with complement (see below).

When HL60 cells were pretreated with varying amounts of H₂DIDS before Cl⁻/glucanate exchange, the cells did not become significantly alkaline (Fig. 1B). The initial pHi of the H₂DIDS-treated HL60 cells (pHi 7.07 ± 0.09) is lower than untreated HL60 cells, suggesting that a still-beneath-inhibitable transport process is required to maintain normal steady state pHi. In any case, inhibition of substantial alkalization by H₂DIDS (Fig. 1, compare A and B) further supports the notion

| Cell line                  | pHi    |
|---------------------------|--------|
| HL60                      | 7.27 ± 0.06 |
| HL60 (CO₂-conditioned 1 h) | 7.39 ± 0.07 |
| HL60 (CO₂-conditioned 2 h) | 7.53 ± 0.09 |
| HL60 RV⁺                  | 7.52 ± 0.03 |
| HL60 Cl⁻–free             | 7.65 ± 0.09 |

3 S. Dzekunov, J. Weisburg, D. A. Scheinberg, and P. D. Roepe, manuscript in preparation.
Cells were attached to glass via the Becton Dickenson “Cell Tak” method (see “Experimental Procedures”). After perfusion with HBSS for 5 min, pHi was monitored, and isotonic Cl-/gluconate exchange was performed (first arrow at 250 s). A, this protocol elevates pHi due to the influx of HCO₃⁻ coupled to Cl⁻ efflux via the anion exchanger present in these cells (described in Ref. 18). B, alkalinization in H₂DIDS-treated HL60 is markedly reduced under similar conditions, due to inhibition of the Cl⁻/HCO₃⁻ exchanger by H₂DIDS. Pretreatment with the stilbene also appears to lower resting pHi slightly. After verifying that alkalinization is reasonably stable for approximately 30 min under each condition, cells were titrated to pH, 6.80, 7.20, and 7.60 (second, third, and fourth arrows, respectively) to calibrate internal BCECF response for the same cell (see Refs. 24 and 25). Data shown in these panels are representative of many experiments (n ≥ 12 for each cell type) that were averaged for different cells to produce the data shown in Table I.

that alkalinization upon Cl⁻ substitution is largely due to reverse Cl⁻/HCO₃⁻ exchange mediated by the Cl⁻/HCO₃⁻ exchanger present in these cells (18).

Resistance of Alkaline HL60 Cells to CMC in Cl⁻-free HBSS—Monoclonal antibody M195 reacts with an antigen typically expressed at a density of about 10,000 sites/cell (GP 67). HL60 cells with elevated pHi due to isotonic Cl⁻ substitution (Fig. 2A, gray bars) were resistant to M195 IgG₁-mediated CMC as compared with HL60 cells suspended in normal HBSS (Fig. 2A, open bars). The degree of resistance for the alkaline cells is similar to the degree of resistance seen previously for the MDR variant RV⁺ cells (Ref. 4 and Fig. 2A, solid bars), which also exhibit an elevated pHi relative to control HL60 cells (Table I). The differences in killing for the alkaline versus control cells are most prominent at lower concentrations of complement and antibody. This suggests that resistance can be overcome with increasing antibody.

HL60 cells with elevated pHi in Cl⁻-free HBSS were also more resistant to a wide range of effective concentrations of an IgM (M31) that recognizes the carbohydrate epitope Lewis X (CD15), in the presence of human complement (Fig. 2B). Again, the efficiency of M31-mediated CMC for HL60 in Cl⁻-free HBSS (Fig. 2B, gray bars) closely resembled that observed for the MDR RV⁺ cells (Fig. 2B, solid bars).

HL60 cells were next pretreated with varying concentrations of H₂DIDS to test if the observed resistance to CMC was due to the Cl⁻/HCO₃⁻ exchange-dependent increase in pHi and not merely to the absence of Cl⁻. In these experiments, the concentration of M195 or M31 that yielded the approximate LD₅₀ (determined in the previous experiment, shown in Fig. 2) was used along with either rabbit complement or human serum, respectively. On average, 44% HL60 cells in HBSS were killed by CMC (Fig. 3A, open bars), whereas only 8% of the HL60 cells in Cl⁻-free HBSS (Fig. 3A, solid bars) were killed by CMC at this dose of antibody. HL60 cells pretreated with 1 mM H₂DIDS before being resuspended in Cl⁻-free HBSS (Fig. 3A, stippled bars) showed a sensitivity similar to that of HL60 in HBSS. Control experiments in which HL60 cells in Cl⁻-free HBSS and treated with only 1 mM H₂DIDS, using medium lacking either complement or antibody (heavy stippled and striped bars, respectively; Fig. 3A), were not killed efficiently showed that the increased cytotoxicity was not due to treatment with H₂DIDS in and of itself (Fig. 3A, compare third, fourth, and fifth bars from the left). Similar relative results were also obtained at lower H₂DIDS concentrations, where the effects of the stilbene are predicted to be even more specific to Cl⁻/HCO₃⁻ exchanger proteins (not shown). A similar reversal of resistance to CMC was seen for H₂DIDS-treated HL60 in Cl⁻-free HBSS that were exposed to M31 IgM antibody and human complement (Fig. 3B).

Elevation of pHi by CO₂ Conditioning Also Results in Resistance to CMC—We also elevated the pHi of control HL60 cells by conditioning them to a 10% CO₂ atmosphere and then placing them back in a 5% CO₂ environment in the presence of fresh pre-equilibrated RPMI medium (see Ref. 2 for a discussion of CO₂ conditioning). Conditioned cells placed back in 5% CO₂ for 2 h were then exposed to M195 and complement and found to be significantly resistant to complement killing at a low doses of M195 antibody (Fig. 4B). The HL60 cells that were grown continuously at 5% CO₂ were effectively killed in a dose-dependent manner as before. Importantly, the degree of resistance to CMC after CO₂ elevation of pHi was time-dependent, as was the extent of alkalinization (see “CO₂ Conditioning Causes Alkalinization of pHi in HL60 Cells”). When the 10% CO₂-conditioned HL60 cells were placed in 5% CO₂-equilibrated medium for 1 h, the cells were less resistant to CMC relative to HL60 cells that were pulsed for 2 h in 5% CO₂ (Fig. 4A). In cells pulsed for 2 h (Fig. 4B), observable resistance extended to higher dosages of M195. Thus, more alkaline pHi appears to result in higher levels of resistance to complement-mediated killing (compare Table I, Fig. 4). These data show that the resistance to CMC after alkalinization can be observed in cells alkalinized in four different ways (chemotherapeutic drug selected with MDR expression; MDR gene transfection (4); Cl⁻...
substitution; and CO₂ pulse) using either human or mouse immunoglobulins of IgG and IgM isotypes and utilizing either rabbit or human complement.

Kinetics of Modulation and Internalization of HL60 and RV⁺ Cells Are Similar—Since CMC is dependent first on antibody binding and retention on the cell surface, especially in the case of IgG antibodies, we wanted to ensure that the resistance of the RV⁺ cells was not due to insufficient numbers of antibodies binding or due to changes in modulation of the immune complex after binding with consequent internalization. We found that multidrug-resistant HL60/RV⁺ cells with elevated pHᵢ bind nearly twice as much ¹²⁵I-HuM195 antibody relative to parental HL60 cells, regardless of the length of time cells are incubated with the antibody. These data confirm (in a more quantitative fashion) our earlier conclusion that RV⁺ cells display more cell surface antibody binding sites relative to parental HL60 cells, extrapolated from indirect flow cytometry measurements (4). The kinetics of cellular internalization of the antibody (revealed as ¹²⁵I counts/min localized to the cell pellet) was similar for both cell lines, although RV⁺ cells initially bound greater levels of HuM195. Thus, importantly, any defect in initiation of the terminal complement cascade (resulting in observed resistance to CMC) cannot be due simply to reduced binding of relevant antibody to the MDR cells; on the contrary, RV⁺ cells bound more immunoglobulin.

Kinetics of MAC Formation Is Delayed in Cells with Elevated pHᵢ—Tschopp et al. (6) previously noted that C9 polymerization required at least 10 min for completion under conditions similar to those used in flow cytometry experiments. Thus, to test whether elevation in pHᵢ alone, without MDR overexpression, affects binding of the MAC to the target cell membrane, control HL60 cells were first CO₂-conditioned and placed back into 5% CO₂ for 2 h to elevate pHᵢ as described above. Then MAC formation was measured by flow cytometry using an antibody that binds to a neoepitope found on C9 only after binding to C8 or polymerizing to other C9 to form a mature MAC (Fig. 5). The alkaline HL60 cells exhibited a delay in peak MAC binding as well as a marked reduction in MAC density relative to control cells. The viability of the HL60 cells at 20 min was 49%, while the “CO₂-pulsed” HL60 cells had 84% viability. The same experiment was repeated using P-GP-expressing RV⁺ cells (not shown). Over 30 min, mean peak channels for the HL60 cells ranged from 3.3 to 7.3, while the RV⁺ cells had mean peak channels of 2.6–3.9. The HL60 cells had a viability of 43% at the 20-min time point, while RV⁺ cells were 75% viable.

Monitoring BCECF Leakage as a Measure of Functional MAC Pore Formation—Binding of a conformationally sensitive antibody is informative but does not determine whether the MACs that are formed are functional. The electrophysiological assessment of MAC pore transport properties can be problematic. Therefore, we explored the possibility of following diffusion of a molecule with an effective radius near 2–3 nm (slightly below the upper limit pore diameter of a minimal (C₅b-C₈)(C₉)₉ full MAC; see Ref. 3) as an indication of functional pore formation. We focused on the frequently utilized fluorescence indicator BCECF. The acetoxy methyl ester derivative of BCECF (BCECF-AM) is neutral and rapidly diffuses into living...
FIG. 3. Complement-mediated cytotoxicity on HL60 cells before (open bars) or after isotonic Cl− substitution to elevate pH, (solid bars) or after isotonic Cl− substitution after pretreatment with H2DIDS (gray bars). M195 (A) or M31 (B) was the antibody used to initiate CMC. Cells exposed to Cl−-free conditions but not H2DIDS (solid bars) are alkaline (Table I) and resistant to CMC, whereas those also pretreated with H2DIDS (lightly stippled bars) are not. Since treatment with H2DIDS in the absence of an initiated CMC pathway (e.g. in either the absence of antibody or complement; heavy stippled bars) does not result in effective kill, reversal of resistance by H2DIDS (a potent inhibitor of Cl− /HCO3− exchange in HL60 cells) indicates that the resistance to CMC upon Cl− substitution (solid bar) is due to alkalization caused by the influx of HCO3− under these conditions (see Fig. 1A).

cells, where it is cleaved by cytosolic esterases and obtains a −4 charge. Passive diffusion of converted BCECF from the cytosol of cells with no large membrane pores is very slow (at most, 5%/h under constant perfusion conditions; see Ref. 2), yet importantly, we do observe significant differences in the effective quantum yield of fluorescence for intracellular versus extracellular BCECF (see Fig. 6A). Moreover, since the fluorescence excitation spectrum is complex, it is reasonable to anticipate that monitoring multiple peaks representing different transitions that respond differently to changes in solvent polarity is feasible. Thus, similar to other studies using a “continuous monitoring of fluorescence” approach (9) but using constantly mixed suspensions of BCECF-preloaded cells (see "Experimental Procedures"), we followed the loss of intracellularly trapped BCECF via a change in the quantum efficiency of 439-nm excitation (Fig. 6A) as a measure of MAC pore formation. Upon the addition of antibody and complement proteins (serum corresponding to ≤15 µg/ml C5, C6, C7, and C8 and approximately 12 µg/ml C9 final concentration)4 to cells, loss of intracellularly trapped BCECF (seen as an increase in 439-nm excitation efficiency for the continuously mixed cell suspension) begins within 12–13 min (Fig. 6A; see also Table II). This time is similar to the time at which the first indications of altered cell viability by trypan blue exclusion are evident (data not shown, but see Ref. 4). By physically separating cells from the medium at various times after the addition of complement and accounting for scatter from the cell suspension (not shown) we are able to conclude that a 2.0–2.5-fold change in 439-nm excitation quantum efficiency accompanies the release of trapped BCECF from these cells and that virtually all BCECF is released within 30 min at this concentration of complement and antibody (see Ref. 9 for similar calculations). No change in 439-nm BCECF response is seen for the continuously mixed suspension when an equivalent amount of heat-inactivated complement is added (Fig. 6A, bottom trace) or when nigericin is added to intentionally change pH (not shown), further supporting the conclusion that these data illustrate formation of large functional (>2.5-nm diameter) pore MACs that accommodates the diffusion of BCECF.

As described under "Experimental Procedures," the BCECF leakage curve may be numerically fit to deduce key parameters. Differentiation converts the computed sigmoid to a gaussian, and the amplitude of the gaussian (defined as A in Equation 1) represents the maximal rate of loss (Vmax) of the BCECF (slope of the sigmoid at its midpoint; cf. Fig. 6A). The characteristic width of the gaussian (σ2) is the time it takes for all BCECF to be released after pores are formed (time of transition between the two steady states), and the difference between t0 (time of the addition of complement, denoted by the arrow in Fig. 6A) and tm (time at which Vmax is computed, i.e. the midpoint of the sigmoid) is defined as the "delay time" (D) or mean time required for fully BCECF-competent channels to form “complete MACs” (Table II).

To test this method and our analysis, we performed multiple experiments under identical conditions but varying the concentration of complement (Fig. 6B). Titration with reduced amounts of complement (in the presence of saturating antibody) significantly shifts the position of the midpoint of maximal change in 439-nm BCECF fluorescence to the right (i.e. increases the value of D, which reflects a decrease in the rate of pore formation). In contrast, only mild effects are found for Vmax, suggesting (as expected) that although the rate of forma-

4 The approximate concentrations of complement proteins are upper limit estimates (41). For C5b(C6,7,8,C9) (n = 9–18) MAC stoichiometry, the C9 subunit is by far the limiting component.
pH, and Multidrug Resistance Regulate Complement Activity

**Fig. 4.** Comparison of CMC for HL60 grown continuously at 5% CO₂ (open bars), RV⁻ grown at 5% CO₂ (solid bars), and HL60 “pulse-elevated” in pH, by 10% CO₂ conditioning followed by placement at 5% CO₂ (stippled bars) for either 1 (A) or 2 h (B). Relative alkalization by this method is dependent upon the time at 5% CO₂ (see also Ref. 2) as is the relative resistance to CMC.

**Fig. 5.** Flow cytometric analysis of MAC pore formation (kinetics) using an antibody that recognizes the neoepitope on C9 during mature MAC formation but not the free C9 component. Normal HL60 cells had an increasing mean peak channel (functional MACs) over time. The CO₂-pulsed HL60 cells showed no increase in mature MAC binding during the same time period. Mean peak channel correlates with the number of C9 neoepitopes per cell.
complement proteins (suspensions of HL60 cells preloaded with BCECF-AM to which normal complement (from treated under identical conditions but exposed to variable levels of 439-nm fluorescence data obtained for aliquots of control HL60 cells min after the addition of complement proteins. Notably, D439 values are significantly delayed for RV⁺ relative to HL60, suggesting a significant increase in the rate of formation of BCECF-conducting MAC pores. Similarly, alkaline HL60 also exhibits increased D values (see "Results").

Experimental Procedures. Data shown are representative of dozens of experiments. In general, at this dose of complement, BCECF leak "delay time" (here and in Ref. 4) was added 100 s after the addition of M31 antibody (antibody added at the arrow). In each case, $10^6$ cells preloaded with BCECF-AM for 30 min were used (see "Experimental Procedures"). The sigmoidal data (top curve) is easily fit to Equation 1 as described under "Experimental Procedures." Data shown are representative of dozens of experiments. In general, at this dose of complement, BCECF leak (revealed as the increase in excitation efficiency) began within 12–13 min after the addition of complement proteins. $B$, representative 439-nm fluorescence data obtained for aliquots of control HL60 cells treated under identical conditions but exposed to variable levels of complement (from left, serum complement that includes 12, 6, or 3 μg/ml C9). Upon diluting complement 2-fold, the time before the appearance of 439-nm changes (illustrating formation of full MACs and loss of intracellular BCECF) increases substantially. A plot of computed "delay time" (D) for these curves and others (versus complement concentration) yields a quadratic relationship (not shown, but see data in Table II) that plateaus near 10 min.

**TABLE II**  
Summary of curve fitting analysis for 439-nm BCECF data

See "Experimental Procedures" for a description of the mathematical analysis. Values are averages of three separate experiments with different batches of antibody and complement. The percentage value for $\sigma^2$ and $V_{\text{max}}$ is relative to the control HL60 at 12 μg/ml C9. Notably, D439 values are significantly delayed for RV⁺ relative to HL60, suggesting a significant increase in the rate of formation of BCECF-conducting MAC pores. Similarly, alkaline HL60 also exhibits increased D values (see "Results").

| Cell line        | Antibody | [C9] | D439 | $\sigma^2$ | $V_{\text{max}}$ |
|------------------|----------|------|------|----------|-----------------|
|                  |          |      |      | %        | %               |
| HL60             | huM195   | 0    |      |          |                 |
| HL60             | huM195   | 12   | 912  | 105      | 9.5             |
| HL60             | huM195   | 6    | 1445 | 205      | 92              |
| HL60             | huM195   | 3    | 2345 | 423      | 122             |
| RV⁺              | huM195   | 12   | 1100 | 310      | 32              |
| Alkaline HL60    | huM195   | 12   | 1070 | 260      | 25              |

**FIG. 6.** A. 439-nm BCECF fluorescence data for continuously mixed suspensions of HL60 cells preloaded with BCECF-AM to which normal complement proteins (upper curve) or heat-inactivated complement (lower line) was added 100 s after the addition of M31 antibody (antibody added at the arrow). In each case, $10^6$ cells preloaded with BCECF-AM for 30 min were used (see "Experimental Procedures"). The sigmoidal data (top curve) is easily fit to Equation 1 as described under "Experimental Procedures." Data shown are representative of dozens of experiments. In general, at this dose of complement, BCECF leak (revealed as the increase in excitation efficiency) began within 12–13 min after the addition of complement proteins. B, representative 439-nm fluorescence data obtained for aliquots of control HL60 cells treated under identical conditions but exposed to variable levels of complement (from left, serum complement that includes 12, 6, or 3 μg/ml C9). Upon diluting complement 2-fold, the time before the appearance of 439-nm changes (illustrating formation of full MACs and loss of intracellular BCECF) increases substantially. A plot of computed "delay time" (D) for these curves and others (versus complement concentration) yields a quadratic relationship (not shown, but see data in Table II) that plateaus near 10 min.

**DISCUSSION**

Resistance to CMC is modulated by P-GP overexpression (here and in Ref. 4); therefore, a suitable molecular explanation for this phenomenon must take into account the effects on agents acting primarily on or in the cell plasma membrane (e.g. antibodies binding to the membrane surface and/or initial assembly of the MAC or MAC pore function). Models envisioning that P-GP is an active efflux pump for drugs (1, 3) or lipids (30) or is a "vacuum cleaner" (1) do not easily account for the observed resistance to CMC.

Conversely, significant changes in pH and $V_m$ have frequently been observed in MDR cells, are due to P-GP overexpression (2, 9, 10), and more easily explain CMC resistance. Although two chemotherapeutic conditioned MDR cell lines have been reported to have normal pH (reviewed in Ref. 12), to our knowledge, every MDR cell line that is MDR purely via overexpression of P-GP (e.g. a "pure" phenotype not further complicated by exposure to chemotherapeutic drug) is alkaline, depolarized, or both (2, 12). These changes are sufficient to explain the relatively low levels of chemotherapeutic drug resistance mediated by P-GP overexpression alone (2). Moreover, these changes in pH (or $V_m$) might form the basis for a more reasonable explanation for resistance to CMC, since significant perturbations in these parameters could influence events that occur at the membrane during initiation of the complement cascade (e.g. assembly of the MAC). To test this idea, in the present study we devised two methods for stably elevating pH in control HL60 cells over a period of time sufficient to measure CMC. We then altered their pH to alkaline values that approximate those observed for the P-GP-overexpressing MDR derivative HL60 RV⁺ (4). These artificially alkaline HL60 were found to be as CMC-resistant as were RV⁺ cells. Moreover, the degree of alkalinization appeared to be related to the degree of resistance. Resistance to CMC was also independent of the mechanism used to alkalinize the cells (e.g. CI⁻ substitution
versus CO₂ pulse versus P-GP overexpression (4)). To firmly test that data from the Cl⁻ substitution experiments indicated that elevation of pHᵢ and not simply loss of Cl⁻ caused resistance to CMC, the effect of Cl⁻ removal in the presence of the stilbene H₂DIDS (to inhibit intracellular alkalinization via reverse Cl⁻/HCO₃⁻ exchange) was examined, and the alternative less invasive CO₂ conditioning technique was also used. Similar resistance to CMC was observed when pHᵢ was elevated via CO₂ pulse, regardless of the specific antibody isotype, antigen type, or species of complement. Thus, elevated pHᵢ in MDR cells (caused by P-GP overexpression) is the most likely explanation for the phenomenon of resistance to CMC.

Two exchangers present in most eukaryotic cell plasma membranes, anion exchanger (Cl⁻/HCO₃⁻ exchanger) and Na⁺/H⁺ exchanger, are vital to pHᵢ homeostasis. In cells that express P-GP, most of the available data suggest that observed alkaline pHᵢ is probably not due to disruption of Na⁺/H⁺ exchanger but to dysregulation of Cl⁻-dependent pHᵢ homeostasis (24). Another hypothesis for the function of P-GP is based on observations of altered ATP transport in MDR cells (31). In this model, ATP transported by P-GP could activate a purinergic receptor/G protein signal transduction pathway that could then regulate ion channels (32). External ATP can increase [Na⁺], and induce biphasic pHᵢ changes characterized by transient acidification followed by significant and sustained alkalinization (33, 34). However, ATP-induced alkalinization of pHᵢ is thought to be due to activation of the amiloride-sensitive Na⁺/H⁺ exchanger, which, as mentioned, is probably not responsible for P-GP-mediated increases in pHᵢ (16, 24). Regardless, via whatever mechanism, elevated pHᵢ caused by overexpression of P-GP may lead to more and different phenotypic alterations in MDR cells than was previously believed to be the case. Resistance to CMC is one example of this. Variability in these phenotypic characteristics for different cell types might help to explain the wide heterogeneity in MDR phenotypes observed to date.

For example, in another recent study (35) an additional effect of P-GP overexpression-induced elevations in pHᵢ was found, namely alterations in the kinetics of the apoptotic cascade for Chinese hamster ovary fibroblasts. Rather dramatic decreases in pHᵢ occur very early on in apoptosis and may be casual or permissive for subsequent events in the cascade (see Ref. 35

**Fig. 7.** Size exclusion chromatography of intact MACs purified from HL60 plasma membranes via the procedure of Ware et al. (28). A, the ¹²⁵I-C9 elution profile on HL60 cells in HBSS; B, the ¹²⁵I-C7 elution profile on HL60 cells in HBSS; C, the ¹²⁵I-C9 elution profile on HL60 cells in Cl⁻-free HBSS; D, the ¹³¹I-C7 elution profile on HL60 cells in Cl⁻-free HBSS for representative columns. In each case, the open squares summarize data for membranes isolated under conditions where MACs are expected to be present (see "Experimental Procedures" and "Results"), and the open diamonds represent control data for uncomplexed (free) complement proteins where MACs are not found. Free C9 and C7 (via either ¹²⁵I or ¹³¹I data) elute near fractions 45–47, whereas complexed C9 and C7 elute in fractions 28–32. By knowing specific activity and ratioing ¹²⁵I versus ¹³¹I, (C5b678a9b):C9 stoichiometry can be computed.
and references within). Thus, pH$_i$ dysregulation caused by P-GP overexpression might indeed be expected to perturb the kinetics of the apoptotic cascade, at least for some cells. Therefore, the consequences of P-GP overexpression should be viewed more broadly when discussing a variety of clinically relevant resistance phenomena. The protection that overexpression of the protein affords to different cell types under different conditions appears to cover a much wider range of cytotoxic situations than initially anticipated. From the clinical perspective, this is of course extremely disappointing. Therapies designed to circumvent P-GP function through modulating drug distribution (but still dependent upon CMC or efficient induction of apoptosis) may not circumvent a MDR phenotype as efficiently as initially anticipated.

With regard to the molecular mechanism whereby elevated pH$_i$ confers CMC resistance, we find that MDR HL60 cells and alkaline HL60 cells have similar binding kinetics for specific antibody at their surface and that RV$^+$ actually binds more antibody; thus, resistance to CMC is not due to a reduced number of antibody sites or reduced ability of antibody to bind (which would provide fewer “anchors” for assembly of complement proteins and subsequent cell lysis). However, flow cytometry utilizing a neo-C9 epitope suggested that the density of functional MACs on the cell surface might be reduced. By analyzing the behavior of 439 BCECF fluorescence traces, we are able to firmly conclude that the rate of formation of complete MAC pores (C5b-C8)$_n$(C9)$_n$ ($n = 9–18$) is significantly reduced in the RV$^+$ and alkaline HL60 cells. Pore formation is slightly faster in alkaline HL60 compared with RV$^+$, suggesting that additional changes in RV$^+$ due to continued selection upon chemotherapeutic drug may also contribute to defects in MAC assembly. One possibility for these additional effects could be altered membrane lipid composition, which appears to occur in some cells upon continued exposure to chemotherapeutic drugs (30). Membrane changes caused by chemotherapeutic drug selection could additionally affect MAC assembly via perturbing electrostatic interactions that appear to be required for insertion of C9 and other subunits (for example, changes in sphingolipid/phosphatidylcholine ratios could alter surface insertion of C9 and other subunits (for example, changes in sphingolipid/phosphatidylcholine ratios could alter surface)

Along with an apparent reduced rate of MAC formation, the BCECF leak data also suggest a slower rate of cellular BCECF release (reduced $V_{max}$) once pores are formed for the MDR and alkaline HL60 cells. Two explanations could conceivably account for these results: 1) a decreased number of complete MAC pores are assembled on the cell surface, or 2) the pore size of MACs for RV$^+$ and alkaline cells is smaller. By dual isotope labeling experiments with purified complement components C7 and C9, we conclude the subunit stoichiometry of MACs for HL60, RV$^+$ and alkaline HL60 is similar. However, more dramatically, quantitation of C7 revealed that there was a clear, significant decrease in the number of MACs assembled on the membrane of RV$^+$ and alkaline cells. This result supports that obtained by flow cytometric analysis with a C9 antibody that binds to a conformationally sensitive “functional” epitope expressed in the assembled MAC. Nonetheless, a slight difference in C9 content is noted, and this could conceivably contribute to the $V_{max}$ effect found for RV$^+$ and alkaline HL60. Although a one-C9 subunit-altered stoichiometry could conceivably affect MAC pore diameter by as much as 9–10%, and although a smaller pore diameter would be consistent with a decreased $V_{max}$, the large perturbations in $V_{max}$ (70–75%) we observe are more easily explained by the observed significant decrease in pore number.

Interestingly, Andrews and colleagues have observed that the pore-forming protein from Trypanosoma cruzi, which was found to be immunologically related to human C9 forms “pores” (or “complement channels”) readily at lower pH but inefficiently at higher pH (36). This observation is similar to those summarized in the present paper, wherein complete C9-containing pores are not formed as efficiently at higher pH.

Effects of extracellular pH on the activation or the pore forming abilities of complement have also been observed, but these are probably due to completely different mechanisms. Human C-reactive protein is known to activate the complement cascade upon reaction with the complement component C1q (37). This activation is dramatically augmented when C-reactive protein is complexed with a suitable ligand, such as phosphocholine-containing (38) and polycation-containing ligands (39). Miyazawa and Inoue (37) observed that in the absence of phosphocholine-containing materials or polycations, the complement cascade could be activated by C-reactive protein at mildly acidic conditions (optimal at pH 6.3) in the presence of negatively charged surfaces. Thus, both pH$_i$ and pH$_o$ are capable of regulating CMC.

Esser et al. (40) have reported that the MAC is capable of physically reorganizing the lipid bilayer during assembly and that the forming MAC affects polar and nonpolar regions of the bilayers at different stages of its assembly. They suggested that C5b-7 interacts strongly with charged regions of the bilayer and may penetrate slightly into the hydrophobic region, while C5b-8 and C5b-9 penetrate more deeply into the membrane. Esser et al. (40) thus concluded that during MAC formation the terminal components undergo dramatic conformational change that allows specific binding sites for phospholipids. Based on the results in this paper, we extend this hypothesis to suggest that the conformational changes are highly pH$_i$-dependent or are dependent upon the magnitude of plasma membrane $\Delta$PH.

Finally, as interesting as these effects of pH$_i$ are, we note that cells overexpressing human MDR 1 typically exhibit decreased $V_m$ as well as elevated pH$_i$ (Ref. 2 and references therein). Possible additional effects (if any) of membrane depolarization in conferring CMC resistance should also be investigated.

In sum, data in this paper and other recent studies (e.g. Refs. 2 and 35) illustrate that models for P-GP function must accommodate a wider array of resistance phenomena than originally anticipated. We believe the altered partitioning model for P-GP (11, 12) is particularly helpful in this regard. It is a useful framework for addressing both CMC resistance phenomena described within as well as apoptotic cascade effects (35) and the thermodynamic and kinetic riddles associated with altered drug partitioning in MDR cells (reviewed in Ref. 12). Further inspection of the model’s implications should continue to resolve clinical questions.

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pH\textsubscript{i} and Multidrug Resistance Regulate Complement Activity