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Changes in plasma membrane phospholipids inhibit antibody-mediated lysis

David T. Harris *

Dept. Immunobiology, P.O. Box 245221, University of Arizona, Tucson, AZ 85724, United States

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ABSTRACT

A variety of mechanisms have been proposed to explain how tumors evade immune destruction. This work has identified one such mechanism that determines susceptibility to immune lysis; membrane phospholipid composition altered susceptibility to antibody plus complement (Ab + C)-mediated lysis. Effects on antibody plus complement-mediated lysis were correlated with levels of major histocompatibility complex (MHC) molecules but not inherent resistance to complement damage. This cellular mechanism could be a means by which tumor cells escape immune detection and destruction.

1. Introduction

Tumor cells grow progressively in vivo despite the host's immune response; which include tumor-specific cytotoxic T lymphocytes (CTL; 1), tumor-specific antibodies and natural killer (NK) cells [1–14]. Many investigators have tried to identify unique tumor cell characteristics correlated with susceptibility to immune cytolysis, starting with differences in tumor cell composition and metabolism. For example, Mandel and Clark [15] studied the fatty acid composition of tumor cells in an attempt to correlate plasma membrane fluidity with changes in either antibody plus complement (Ab) or CTL-mediated lysis of the cells, with no effect. Shinitzky et al. [16] analyzed tumor cell membrane viscosity, noting that when plasma membrane viscosity increased the cells became better immunogens. Finally, Schlager and Ohanian [17] and Schlager [18] correlated the ability of tumor cells to incorporate fatty acids into complex membrane-bound lipids with changes in susceptibility to Ab- and CTL-mediated lysis. Unfortunately, these studies were not able to specifically and uniquely alter these individual parameters to determine specific effects on cytolysis. More recently, other investigators have found additional roles for phospholipids in inducing and preventing apoptosis, immune regulation, tumor progression and drug resistance [19,20].

In addition, an earlier study examining tumorigenic esophageal cell lines demonstrated a correlation between tumor cell lipids and the ability of a tumor-specific antibody to bind to its tumor antigen [21]. These results implicated tumor cell lipid composition as a potential mechanism for tumor escape from immune destruction. In the current study, we examined the effects of specific alterations in the phospholipid composition of target cells [22–24] on susceptibility to antibody-mediated (Ab + C) cytolysis. Growth of cells in choline analogs significantly changed target cell susceptibility to Ab + C lysis. A correlation of lytic sensitivity with changes in MHC levels was observed. It is proposed that membrane phospholipid composition may significantly alter a cell's susceptibility to immune destruction, allowing tumors to escape immune recognition and lysis.

2. Materials and methods

2.1. Mice

Balb.c and Balb.k inbred mice were used in these studies. All animal studies were reviewed, approved and conducted according to AAALAC guidelines.

2.2. Phospholipid supplementation of LM cells

Cultures of LM cells (fibroblast of C3H origin) were grown at 37C in Higuchi's medium [25] containing 20 mM HEPES buffer, pH 7.4. This medium is a chemically-defined, lipid-free medium that supports growth of LM cells indefinitely in the absence of serum when choline (C) is present. Cells to be grown in the presence of C analogs were harvested, washed and resuspended in the desired medium. The following C analogs were utilized: ethanolamine (E), monomethylethanolamine (MME), dimethylethanolamine (DME), L-2-amino-1-butanol (AB) and 3-aminopropanol (AP). Analogs were added at 40 μg/ml and except for AB, were obtained from Eastman

Abbreviations: Ab + C, antibody + complement; AB, L-2-amino-butanol; AP, 3-aminopropanol; C, choline; CVF, cobra venom factor; DME, dimethylethanolamine; E, ethanolamine; MME, monomethylethanolamine.

* Fax: +1 520 626 2100.
E-mail address: davidh@email.arizona.edu

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Kodak Company. AB was obtained from Aldrich Chemical Company. The cells were grown for 3 days in the supplemented medium prior to use in the assays [22].

2.3. Phospholipid and fatty acid analyses

Cells grown in choline were incubated for 5d in the presence of 5 μCi/ml of ^{32}P-orthophosphate prior to exposure to C analogs. Cells were then exposed to C analogs for 3d, keeping the ^{32}P concentration constant. Cells were washed 2× in Ca^{2+}, Mg^{2+}-free PBS and whole cell lipids extracted by the method of Bligh and Dyer [26]. The phospholipids were separated by 2-dimensional thin layer chromatography (TLC) on silica gel H (250 μm), made visible by autoradiography [27], and counted in Omnifluor–toluene–Triton X-100–H_2O (4%–2:1:0.2) scintillation fluid in a scintillation counter. Phospholipids were identified by comparison with known standards. The solvent system consisted of chloroform–methanol–H_2O (87:31:5) in the first dimension and *N*-butanol-glacial acetic acid–H_2O (80:26:26) in the second dimension.

For fatty acids analysis, cells were harvested, phospholipids extracted and separated from neutral lipids on a silicic acid column (0.3 g Unisil in a disposable Pasteur pipette plugged with glass wool). The neutral lipids and phospholipids were eluted with 5 ml of chloroform and 4 ml of methanol, respectively. Fatty acid methyl esters were prepared from the phospholipid fraction by the addition of 2.5 ml of pure methanol and 0.2 ml of H_2SO_4 to samples in Teflon-lined screw-capped tubes. The tubes were flushed with nitrogen, sealed and heated at 80 °C for 2.5 h after which tubes were cooled, 2.0 ml of H_2O and 2.5 ml of pentane was added, and the pentane layer removed for analysis of fatty acid content via gas layer chromatography (OV351 capillary column, 62 m in length, 1 ml/min Helium flow rate, 155–200 °C at 8 °C/min and 201–245 °C at 5 °C/min).

2.4. Antibodies

H-2^d–anti-H-2^k antisera was produced in Balb.c mice following intraperitoneal injection of 2×10^7 Balb.k splenocytes, every other week for 10 weeks. The mice were bled by cardiac puncture and the sera were heat-inactivated at 56 °C, 30 min. The sera was absorbed with Balb.c splenocytes (1×10^7, 2×) prior to use.

The hybrodoma 15.3.1 (monoclonal anti-H-2K^k, [28]) was obtained from the Salk Institute (LaJolla, CA). Culture supernatants were purified by affinity chromatography on protein A columns and adjusted to 1.5 mg/ml prior to use [29].

2.5. Antibody plus complement (Ab + C)-mediated cytotoxicity assays

Target cells were labeled with ^{51}Cr (sodium chromate) and incubated at 4 °C, 30 min with serial dilutions of antisera in microtitre plates. The cells were washed 3× and a 1:10 dilution of rabbit complement (Pel-Freez) was added. The mixture was incubated at 37 °C, 30 min before washing (3×). Residual radioactivity in the cell pellets was determined using a gamma counter. Controls included media, complement and antibodies alone.

2.6. Measurement of complement

Experiments examining the ability of antibody when bound to target cells to deplete complement were performed as follows. Control and lipid altered cells were incubated with anti-H-2K^k monoclonal antibody and complement (rabbit) as described above. The cells were centrifuged (500*g*, 5 min) and the supernatant fluid (containing residual complement) were removed. The supernatant was added to hemolysin-sensitized sheep red blood cells (SRBC) and incubated for 45 min, 37 °C. The mixtures were centrifuged (500*g*, 10 min) and the absorbance of the supernatants was measured by spectrometry (541 nm). The optical density was compared to a standard curve to determine the levels of residual complement. Controls consisted of (a) cells to which no complement was added, (b) cells to which was added complement that had been incubated with antibody at 37 °C without the presence of cells, and (c) cells to which was added unabsorbed complement.

2.7. Antibody-independent complement activation

The ability of complement to induce lesions in target cell plasma membranes (independent of antibody) was examined as follows. ^{51}Cr-labeled cells were placed in 96 well microtitre plates (5×10^4 cells in 50 μl). Rabbit complement (50 μl, 1:5 dilution, Pel-Freez) and cobra venom factor (CVF; 50 μl, 1:5 dilution, Cordis Laboratories) were added, in that order, and the plates incubated at 37 °C. Controls consisted of cells incubated with (a) media alone, (b) complement alone, or (c) CVF alone. Plates were processed and ^{51}Cr release was calculated as described previously.

2.8. FACS analysis

H-2 antigen levels were examined by FACS analysis. The anti-H-2K^k mAb and a FITC-labeled rabbit-anti-mouse IgG antibody (Cappel Laboratories) were used. Samples were analyzed using an Ortho 50H Cytofluorgraph equipped with a 4 W argon laser.

2.9. Statistics

All statistical analyses were performed using Student's *t*-tests ($p < 0.05$) as found in SPSS.

3. Results

3.1. Specific alteration of cell membrane phospholipid composition by choline analogs

Target cells were grown in the presence of choline or one of five analogs (E, MME, DME, AB or AP) to specifically change membrane phospholipid composition. Choline-supplemented media served as the control media and was used for long term maintenance of the cells. As shown in Table 1, these culture conditions resulted in significant and specific alterations in the phospholipid profile of the cells. For AB and AP supplementation, it was possible to significantly introduce an unnatural phospholipid component into the cellular phospholipid profile. Cells grown in E-supplemented media poorly incorporate the analog as previously noted [19]. No significant differences were found in any of the minor phospholipids (e.g., sphingomyelin, phosphatidylserine, phosphatidylinositol, and cardiolipin).

Analyses were also performed to determine if these modifications affected the fatty acid composition of the cells, as this variable has been shown to alter target cell lysis [30]. As shown in Table 2, no significant changes in fatty acid composition due to phospholipid alterations were observed. Fatty acids of the 18:1 variety were the major constituent (>50%) in each one of the analog-supplemented cells. Only minor variations in other fatty acids (16:0, 16:1, 18:0) were observed.

3.2. Membrane phospholipid alterations inhibit Ab + C cytolysis

As shown in Fig. 1, changes in cellular phospholipid composition resulted in significant changes in susceptibility to Ab-mediated lysis using two different antibodies; an H-2^d–anti-H-2^k

Table 1
Phospholipid composition of cells grown in choline analogs.

Supplement[a]	Phospholipid composition (%)[b]									Total %
	PC	PE	PMME	PDME	PAB	PAP	SM	PS/PI	CL	
C	49.8	25.4	1.8	2.1	–	–	10.3	7.4	3.1	99.9
E	44.2	32.5	–	1.2	–	–	9.1	9.2	3.9	100
MME	20.1	13.5	44.6	3.8	–	–	9.6	5.8	2.6	100
DME	13.0	15.3	1.8	53.4	–	–	7.7	6.5	2.4	100.1
AB	39.1	16.1	0.7	0.7	22.5	–	10.6	7.6	2.7	100
AP	25.8	14.8	1.5	–	–	34.9	10.8	8.5	3.7	100

The underline represents the expected phospholipid to contain the highest amount of the added lipid.
[a] Cells were grown for 3 days in C or analogs as described. Cells were ^{32}P-labeled and analyzed by TLC as described. Data represent the combination of three individual experiments and are expressed as percent composition. Standard deviations between experiments for an individual phospholipid class differed by less than 10%.
[b] Cellular membranes were analyzed for the following lipid classes: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylmonomethylethanolamine (PMME), phosphatidyldimethylethanolamine (PDME), phosphatidyl-L-2-amino-butanol (PAB), phosphatidyl-3-aminopropanol (PAP), sphingomyelin (SM), phosphatidylserine/phosphatidylinositol (PS/PI), and cardiolipin (CL).

Table 2
Effect of phospholipid supplementation on fatty acid composition.

Cell	Fatty acid analyses							
Media	16:0	16:1	18:0	18:1	18:2	18:3	22:5	22:6
C	17.6	3.3	12.8	58.9	0.0	0.0	1.3	3.0
E	10.2	3.4	17.2	57.4	0.0	0.0	1.8	4.6
MME	11.2	13.9	18.7	53.1	2.4	0.0	2.5	2.5
DME	10.0	13.0	17.1	50.7	3.2	2.0	3.4	3.4
AB	10.0	13.0	14.4	63.4	0.0	0.0	2.4	5.0
AP	14.4	13.8	20.1	55.9	0.0	0.0	1.4	3.4

Cells were grown in either choline (C) or analogs (E, MME, DME, AB or AP) as described. Fatty acid composition analysis was performed as described. Data represent the percent composition for each individual cell type. Data is representative of three independent experiments.

antisera and a monoclonal anti-H-2K^k antibody. Although absolute levels of lysis differed between the two antibodies, the overall patterns of lysis were consistent. Cells grown in C, E and MME were significantly more susceptible to lysis than cells grown in AB and AP, while the DME-supplemented cells were very resistant to Ab-mediated killing. Slight discrepancies in the absolute rank order of lysis may be due to availability of antigenic sites recognized by each of the antibodies on each of the individual targets (H-2D^k and H-K^k antigens for the antisera vs. H-2K^k antigens only for the monoclonal antibody).

3.3. Possible mechanisms for inhibition of Ab-mediated lysis

Inhibition of Ab-mediated lysis was potentially due to inhibition of Ab binding (inhibition of recognition), a failure of bound Ab to activate complement (inhibition of Ab function) or an intrinsic resistance of the lipid-altered cells to the actions of complement itself (inhibition of lysis).

The ability of Ab, when bound to the phospholipid modified cells, to activate complement was examined, as shown in Table 3. Cells that had been cultured in either C (very Ab-sensitive) or DME (extremely Ab-resistant), were labeled with equal amounts of the monoclonal antibody (determined by binding of iodinated 15.3.1 antibody, data not shown), and the ability to deplete complement was analyzed. Over a wide range of cell numbers used for the depletion assays it was observed that antibody bound to either C or DME supplemented target cells consumed equivalent amounts of complement. Thus, differential activation or inactivation of complement was an unlikely factor in the susceptibility of the cells to Ab-mediated killing.

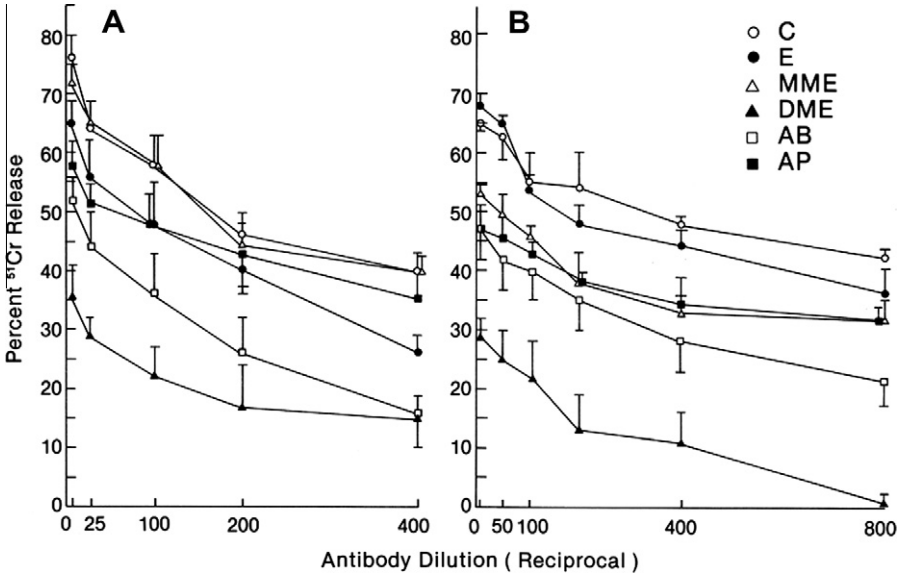

Fig. 1. Ab-mediated lysis of cells grown in choline analogs. Cells grown in choline analogs were analyzed for susceptibility to lysis using either (A) H-2^d–anti-H-2^k antisera, or (B) monoclonal anti-H-2K^k 15.3.1 antibody in the presence of complement. Values are expressed as the percent release ^{51}Cr-radiolabel release. Each data point represents the mean ± SD of three independent experiments.

Table 3
Effect of cellular phospholipid composition on antibody function.

Supplement[a]	Cell number[b]	% Depletion[c]
C	1×10^7	88.0
	1×10^6	80.0
	5×10^5	43.5
	1×10^5	37.3
	5×10^4	37.4
	1×10^4	27.4
	1×10^3	23.1
DME	1×10^7	87.5
	1×10^6	76.8
	5×10^5	50.0
	1×10^5	39.2
	5×10^4	35.7
	1×10^4	25.2
	1×10^3	20.3

[a] Cells were grown in choline or DME.
[b] Cell number used for complement depletion. Cells were coated with saturating amounts of anti-H-2K^k mAb (15.3.1) prior to depletion (1/1600 dilution).
[c] Percent complement depletion was calculated from a standard complement titration curve. Rabbit complement was used at a 1/10 dilution and SRBC were sensitized with a 1/100 dilution of hemolysin.

The intrinsic susceptibility of modified target cells to complement-induced lytic lysis was examined. These experiments used cobra venom factor (CVF) which directly activates C3 in the complement cascade, does not require the presence of antibody, and in the presence of cells will directly mediate lysis [31,32]. The magnitude of lysis (as assessed by ^{51}Cr radiolabel release) and the kinetics of lysis were analyzed (Fig. 2). It was observed that C (very sensitive) and DME (extremely resistant) cell types were highly susceptible to complement-mediated lysis, while AP (intermediate) cells were somewhat resistant at later time points. The kinetics of lysis, for all cell types were similar.

Finally, the levels of the target H-2K^k antigen were analyzed. Cells grown in C, DME and AP were chosen in that these conditions represented the entire range of lytic susceptibility. As seen in Fig. 3, alterations in phospholipid composition significantly affected the levels of H-2K^k antigens, as well as the percentage of cells expressing H-2K^k molecules. DME-supplemented cells (a highly resistant target) exhibited high levels of H-2K^k antigens, while AP-supplemented cells (an intermediately resistant target) was observed to have much lower levels of H-2K^k antigens. The C-supplemented cells, highly sensitive to Ab-mediated killing, were intermediate in the levels of H-2K^k antigens.

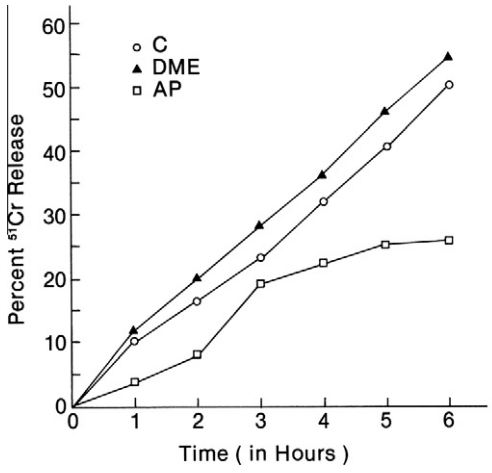

Fig. 2. Effect of cell phospholipid composition on antibody-independent complement-induced lysis. Cells were grown in choline (C), dimethylethanolamine (DME) or 3-aminopropanol (AP). Cobra venom factor-mediated complement activation was performed as described. Results are expressed as the means of six independent replicates. Standard deviations did not exceed 5%. Each time point from 4 h onwards for C and DME were significantly different from AP, but not from each other ($p < 0.05$).

To further examine the role of H-2K^k antigen expression, the C (sensitive) and DME (resistant) populations were sorted into low and high-H-2K^k-expressing subpopulations (upper and lower 20% of cells in each profile). These subpopulations were used as target cells in Ab-mediated killing assays, and compared to unsorted cells (Table 4). Target cells expressing lower levels of H-2K^k antigens were as or more susceptible to lysis as unsorted cells, for either the C or DME cell types. However, target cells expressing higher levels of H-2K^k molecules, regardless of the supplementation, were found to be extremely resistant to Ab-mediated killing (3- to 6-fold as compared to unsorted populations). These results agreed with the hierarchy of target cell susceptibility to Ab lysis of unsorted target cells. That is, the DME population contained a greater percentage of cells with higher H-2 levels (approximately 60%) and was very resistant to lysis. The C population however, possessed fewer cells of this type (approximately 30%) and was more susceptible to Ab-mediated killing.

4. Discussion

Immune responses to viable tumors in animals previously immunized with non-viable tumor cells are well established [1–2], and there is little doubt that malignant cells can be destroyed in vitro by different immune effector mechanisms. Since cancer frequently arises as a clone from a single cell [3], any new or modified genetic information conferring selective growth advantages may be preferentially selected and maintained. These cells can then give rise to the dominant phenotype of the emerging malignancy, expressing traits selected to bypass or thwart host defenses. Mechanisms proposed to accomplish this include "blocking" antibodies [4], tumor antigen shedding [5–6], and tumor-induced host suppression [7]. These mechanisms can be classified as: changes in recognition of the tumor by the immune system; inhibition of the host's various cytolytic mechanisms; and intrinsic resistance of the tumor to immune cytolysis. Immune mechanisms that must be circumvented for progressive in vivo tumor growth include: tumor-specific antibodies and CTL and NK cells [8–14].

Early investigators tried to correlate tumor cell susceptibility to immune lysis with physical characteristics of tumor cells, with mixed results [15–18]. More recently, Li et al. [33] correlated membrane phospholipid changes found in human colorectal cancers with tumorigenesis and metastasis in 49 patients. In these studies phosphatidylethanolamine (PE) content was of particular importance. Dobyznska et al. [34] also by studying human colorectal cancers arrived at a similar conclusion regarding membrane phospholipids and the processes of carcinogenesis and metastasis. In these findings it was the ratio of PC/PE that was found to be most important in malignancy. This group of investigators had reported similar results in an earlier publication as well [35]. Interestingly, phospholipid composition was also found to influence tumor cell resistance to chemotherapeutic drugs [36,37], supportive of the role of these lipids in protecting developing tumor cells from a variety of insults.

In our work, we utilized a model system in which the effect of specific alterations in target cell phospholipid composition could be analyzed for its contribution to cellular susceptibility to immune lysis [19–21]. This system minimized the number of interacting variables in an attempt to discover cellular characteristics that determined target cell susceptibility to immune lysis.

Cellular phospholipid composition was found to induce significant differences in susceptibility to Ab + C-mediated killing (2- to 5-fold). To understand how alterations resulted in the observed

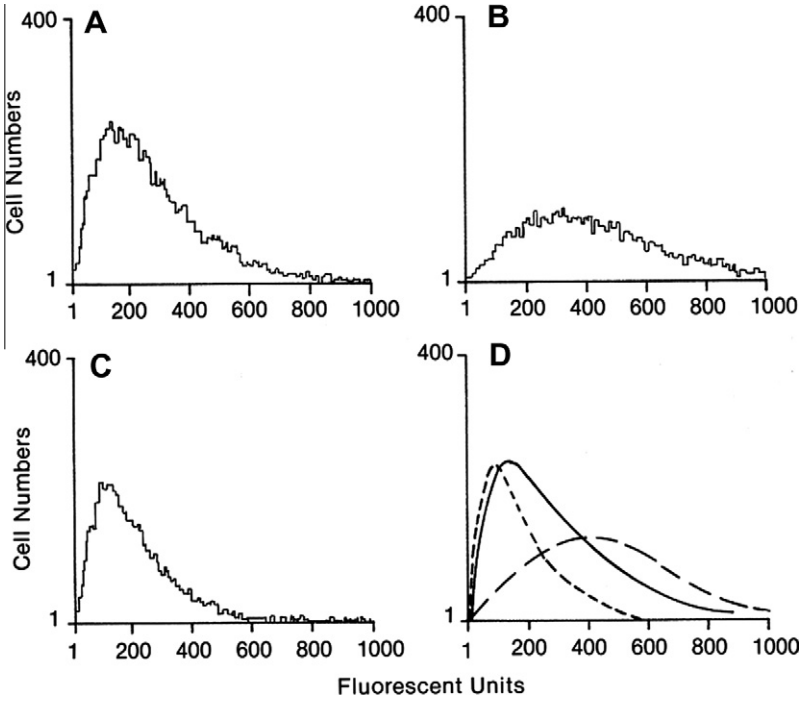

Fig. 3. Effects of phospholipid composition on H-2K^k antigen expression. Flow cytometric analysis using an anti-H-2K^k mAb (15.3.1) was performed as described. Cells were grown in either (A) choline (C); (B) dimethylethanolamine (DME); or (C) 3-aminopropanol (AP). All three analyses are shown in (D) for comparison.

Table 4
Effects of H-2K^k levels on susceptibility to Ab + C.

Supplement[a]	Cell population[b]	% ^{51}Cr release[c]
C	Unsorted	66.0
	Low H-2	55.1
	High H-2	10.0*
DME	Unsorted	39.8
	Low H-2	45.1
	High H-2	12.6*

[a] Cells were grown for 3 days in choline (C) or DME.
[b] Cell populations used in the cytotoxicity assays were either not sorted (unsorted), the lower 20% of the unsorted population (low H-2) or the upper 20% of the unsorted population (high H-2).
[c] Data are presented as the percent ^{51}Cr release of assays performed in replicates of 6. Standard deviations were less than 5%. A representative experiment (of three) is shown.
* Significant from the unsorted population at $p < 0.05$.

differences in susceptibility to immune lysis we investigated whether the differences were due to recognition of the target cell antigens by the Ab, inhibition of Ab function or intrinsic changes in cellular resistance to complement mediated lysis. Experiments analyzing the ability of cell-bound antibody to deplete complement indicated that equivalent complement consumption occurred. Thus, phospholipid composition did not affect complement activation and fixation. If the target cells utilized complement equally, then the basis for the differential levels of lysis could possibly be related to the intrinsic susceptibility of the target cells to complement-induced lysis. However, the different cell types were not differentially susceptible to the lytic effects of complement.

To resolve this dilemma we sorted target cell populations into subsets bearing higher and lower levels of H-2K^k antigens (as the sensitive and resistant populations differed in H-2K^k levels), and examined each population for susceptibility to Ab + C. It was found that subpopulations of cells exhibiting higher H-2K^k levels were extremely resistant to Ab + C lysis (3- to 6-fold). Cells displaying lower H-2K^k levels were as equally susceptible to lysis as the unsorted population. Thus, increased levels of H-2K^k molecules seemed to result in resistance of the target cells to Ab + C. It is well accepted that too little target antigen may result in inefficient killing [38,39]. It has not been shown however, that too much target antigen could have the same effect. Circolo and Borsos [40] inferred that such a phenomenon might occur based on work in an anti-hapten system, but no rigorous proof was offered. The manner in which elevated H-2K^k affected Ab + C was not determined, but complement activation changes when Ab is bound to cells with high H-2K^k seems feasible.

This study examined the role of membrane phospholipid composition on a cell's susceptibility to Ab + C. This aspect was emphasized because it is at the level of the membrane that target structures recognized by Ab are expressed, this is the location where the target cell/Ab interaction is effected, and it is the location at which the cytolytic event is imparted. Thus, any alteration here could have a significant effect on immune destruction. Alterations in phospholipid composition altered susceptibility to Ab + C by modifying H-2K^k levels (which seemed to enable the cells to resist such Ab + C). It is hypothesized that this type of cellular modification may be a mechanism by which tumors could evade immune destruction. It is conceivable that such phospholipid modifications may arise either from the neoplastic transformation process or from immune selection pressures on the developing tumor population. The membrane phospholipid composition may not only affect anti-tumor immune responses [41–43] it may also affect the ability to generate cytolytic responses to viral, bacterial and/or parasitic infections due to a lack of recognition [44–47]. Finally, knowing which alterations make cells more or less recognizable to the immune system may also be of importance in the design of cellular vaccines (e.g., stimulate recognition) and cellular therapies (e.g., escape recognition).

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