Differential recognition and lysis of EL4 target cells by cytotoxic T cells: differences in H-2Kb antigenic density and cytoskeletal proteins

RV Flores and PJ Gilmer

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DIFFERENTIAL RECOGNITION AND LYSIS OF EL4 TARGET CELLS BY
CYTOTOXIC T CELLS: DIFFERENCES IN H-2K\textsuperscript{b} ANTIGENIC DENSITY AND
CYTOSKELETAL PROTEINS\textsuperscript{1}

ROSA V. FLORES\textsuperscript{2} AND PENNY J. GILMER\textsuperscript{3}

From the Department of Chemistry and the Institute of Molecular Biophysics, Florida State University,
Tallahassee, FL 32306

Direct binding of \textsuperscript{125}I-iodinated anti-H-2K\textsuperscript{b} monocl-onal antibody (B8-24-3) to EL4 cells indicated a similar association constant but a 2.7-fold higher H-2K\textsuperscript{b} antigenic density when the EL4 cells were grown in ascites (2.0 $\times$ 10\textsuperscript{6} sites/cell) than in tissue culture (7.5 $\times$ 10\textsuperscript{6} sites/cell). The membrane fluidity of the isolated plasma membrane fractions, measured by using a membrane-sensitive spin label, was essentially identical in the two differently cultured EL4 cells. There were some differences in the cytoskeletal proteins that were isolated from the plasma membrane fractions of the EL4 lines and analyzed by two-dimensional gel electrophoresis. These differences in the H-2K\textsuperscript{b} antigenic density and the cytoskeletal proteins may contribute to the 2.3-fold higher \(V_{\max}\) and the increased binding of the EL4 ascites target cells to allogeneically primed anti-H-2K\textsuperscript{b} cytotoxic T cells.

The factors that modulate immune recognition and lysis of target cells by specific cytotoxic T lymphocytes (CTL)\textsuperscript{4} have been examined in various ways. The serologically detectable, class I major histocompatibility antigens H-2K and -D on the target cell have been shown to be critical for interaction with CTL (1, 2). When the target cell antigen is blocked by an antibody, T cell-mediated lysis of the target cell bearing that antigen is specifically inhibited (1, 3, 4). As the density of the antigens on the target cell is reduced by papain cleavage, the lytic reactivity of the target cells decreases (5), implying a direct correlation between the antigenic density and the ability to be lysed by the CTL.

The interaction between allogeneically primed CTL and target cells was chosen so as to examine the factors that modulate immune recognition and lysis, because the primary antigen on the target cell that is recognized in the allogeneic system is a major histocompatibility complex (MHC) antigen. In contrast, in the virally infected syngeneic system, both the MHC and the viral antigens must be recognized. Recent studies have shown that cloned CTL react not only with the cell with which they were stimulated, but also with a cross-reacting allogeneic cell (6–8). These results suggest that some factors that modulate immune recognition and lysis in the allogeneic system may well apply to the more biologically relevant, but experimentally more difficult, virally infected or chemically modified syngeneic cells.

In the experiments described here, an EL4 target cell line that has differing reactivities with allogeneically primed CTL was compared, depending on the culture conditions of the target cell (9). The CTL are directed primarily at the H-2K\textsuperscript{b} antigen (10). Previous experiments conducted in this laboratory have shown that EL4 ascites target cells are more susceptible to T cell-mediated lysis than EL4 cells grown in tissue culture (EL4 t.c.) (9). The more susceptible EL4 ascites target cell has a 2.2-fold less neuraminidase-releasable cell surface sialic acid than the less susceptible EL4 t.c. target cell (9). This difference probably is due to a neuraminidase activity found in the peritoneal fluid of C57BL/6 mice injected with EL4 ascites cells (9). Minimally, neuraminidase treatment desialylates the H-2\textsuperscript{k} molecules, as demonstrated by twodimensional (2D) gel electrophoresis (11). The effect of different disialylated forms of H-2 molecules on T cell-target cell recognition was also recently explored in this laboratory by using a wheat germ agglutinin-resistant variant of EL4 t.c. (10). Other possible explanations for the difference in susceptibility of the differently cultured target cells to CTL, such as antigenic density, target cell membrane fluidity, and the composition of the cytoskeletal proteins (CSP), were investigated in this study.

MATERIALS AND METHODS

Mice. Six to 16-week-old BALB/c and C57BL/6 mice were obtained from Charles River Laboratory (Wilmington, MA) or from our own breeding colony.

Cell lines. EL4 (H-2\textsuperscript{k}) is a murine lymphocytic leukemia line derived from C57BL/6, and is available from the Salk Institute (La Jolla, CA). EL4 ascites cells were maintained in the ascitic form by passage every 4 to 6 days by injecting a syngeneic C57BL/6 mouse i.p. with 5 $\times$ 10\textsuperscript{6} EL4 ascites cells. Female C57BL/6 were used to grow the EL4 ascites cells for use as targets or stimulators, and males were used for EL4 membrane preparations. EL4 t.c. cells were grown at 37°C in a 5% carbon dioxide incubator and were passed every 2 to 3 days in Dulbecco's modified Eagle's medium (DMEM) (GIBCO No. 430-1600; Grand Island, NY) containing 10% heat-inactivated horse serum (HS) (GIBCO No. 230-6050) and 1% penicillin-streptomycin (PS) (Eli Lilly Nos. 526 & 431, respectively; Indianapo-lis, IN). The differently cultured EL4 cells were passed and main-

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\textsuperscript{3} All correspondence and reprint requests should be addressed to Dr. Gilmer.

\textsuperscript{4} Abbreviations used in this paper: CSP, cytoskeletal proteins; CTL, cytotoxic T lymphocytes; HS, heat-inactivated horse serum; mcAb, monoclonal antibody; PM, plasma membrane; PS, penicillin-streptomycin; t.c., tissue culture; 2D, two-dimensional.
tained independently of each other. A 2D gel analysis of in vitro [35S]methionine-labeled plasma membrane (PM) preparations from EL4 ascites (11) and EL4 t.c. (H-2 region shown in Reference 10) were essentially identical, indicating that the same proteins were isolated. The petri dishes were incubated with rocking at 37°C for 1.5 hr. The velocity of target cell lysis (v) was calculated by the formula:

\[ v = \left( \frac{9}{\mu} \cdot \text{Cr release} \right) \cdot \text{(target cells)} \cdot 100 \quad (1.5 \text{ hr} - \text{lag time}) \]

The values for \( V_{\text{max}} \) and \( K_m \) were determined from a linear regression analysis of the data in an Eadie-Hofstee plot.

The dissociation target cell conjugates was similar to that described previously (9), except that 1) conjugate formation was performed at room temperature by centrifugation at 300 g for 10 min in a swinging bucket rotor, and 2) the T cells were scored for percent T cells bound to target cells (10). The anti-H-2Kb T cells were mixed with fluorescein diacetate-labeled target cells, an effector to target cell ratio of 1:1, 1.8 \times 10^6 cells total in a final volume of 30 \muL. The centrifuged conjugates were resuspended 10 times with a pipette and placed on ice. The resuspended sample (7 \muL) was analyzed under a Leitz epifluorescence microscope with an 1-2 FITC filter, and the T cells bound to a fluorescent target cell were enumerated. A total of 200 T cells were counted for each sample. The standard deviation of the percent conjugates was 3%.

**Membrane fluidity determination.** The PM fraction from EL4 ascites or EL4 t.c. were boiled for 5 min in 10 mM Tris-Cl, pH 7.5, and centrifuged at 87,000 g for 45 min at 4°C. The sediment was resuspended in 10 mM Tris-Cl, pH 7.5, and protein content was determined by a Folin assay (16) with bovine serum albumin used as the protein standard. The (12,3)-methyl iodide, 2,4-dinitrophenylglycine-(2,4,6-tricyclo-3-oxazolidinylxynonyl) (Syva No. 618, Palo Alto, CA), at 4.3 mM in 100 mM ethanol, was divided into aliquots of 2 \muL and transferred to glass test tubes where the solvent was carefully evaporated under the use of nitrogen. Volumes of PM fractions from the differently cultured EL4 cells containing the same amount of membrane proteins were aliquoted into the spin label coated test tubes. The amount of spin label varied slightly from 2.7 to 2.9% (mol/mol) of total lipid. The moles of lipid were calculated under the assumption that EL4 t.c. (H-2 region shown in Reference 10) were prepared from 

\[ V_{\text{max}} \cdot K_m \]

\[ \text{the average m.w. of the lipid was that of dipalmitoylphosphatidylcholine. Test tubes were vortexed and placed in a 37°C water bath for 60 min, with occasional vortexing. Samples were transferred to capillary tubes, and the EPR spectra were obtained from a Varian Model E-12 spectrometer, operated in the X-band frequency range. Temperature in the cavity was varied by heating a stream of nitrogen gas with a temperature controller and measuring the temperature with a thermocouple.} \]

\[ \text{CSP evaluation. The CSP were isolated from the PM fraction as described (17), except 0.2 M phenylmethanesulfonfluoride (Sigma Chemical, St. Louis, MO) was present during the detergent solubilization step. The final sediments from the differently cultured EL4 t.c. were resuspended in 0.5% Nonidet P-40, were analyzed by 2D gel electrophoresis (11).} \]

**Comparison of H-2Kb antigenic density on EL4 ascites and EL4 t.c. cells.** The H-2Kb density on the cells was determined by comparing the binding of [35S]-iodinated anti-H-2Kb (B8-24-3) mAb to EL4 ascites and to EL4 t.c. cells. Radiolabeling of the antibody was performed according to David and Reisfeld (18) except that the reagents were mixed at room temperature and then allowed to react for 20 min on ice. The reaction was terminated upon addition of Na azide. The sample was diluted into transfer solution (16.0% (w/v) sucrose in PBS containing 0.01% bromphenol blue) and layered on a 23 x 1 cm Sephadex G-50 column pre-equilibrated in PBS. Fractions (15 drops) were collected into test tubes containing 200 \muL PBS supplemented with 0.1% gelatin (Knox). The final concentration of gelatin was 0.05%. Fractions containing the radiolabeled antibody were pooled (total volume was 2.0 to 2.5 ml). The iodinated antibody was analyzed by 2D gel electrophoresis, and an autoradiogram demonstrated that only the heavy chain antibody molecule was appreciably radiolabeled (data not shown).

The binding assay was performed in 500-\muL capacity plastic microtiter tubes that had been pre-equilibrated in PBS-0.05% gelatin overnight at 4°C. [35S]-iodinated anti-H-2Kb (50 \muL) at 3.9 \muCi/ml, serially diluted by a factor of 2 to 3 in PBS-0.05% gelatin to 0.1 \muCi/ml, was added to each tube. T cells containing 50 \muL of iodinated antibody plus 25 \muL PBS-0.05% gelatin without any cells were also prepared to assess nonspecific binding of the antibody to the tube. The tubes were incubated on ice for 1 hr, with occasional vortexing. Then 20 \muL of cold irradiated ascites (10 \muCi/ml) in 0.33X PBS) was layered underneath the resuspended cells on ice. The antibody-adsorbed cells were separated from the free
antibody by centrifugation at 300 × G for 10 min at 4°C. All but 40 
μl of the fluid was carefully aspirated off the top of each tube. The 
upper halves of the microcentrifuge tubes were cut so that the bottom 
portion of the tubes would fit into the 12 × 75 mm test tubes for 
gamma counting. All samples were done in duplicate.

Comparison of the H-2K^b density on EL4 ascites and EL4 t.c. cells 
was assessed by a Scatchard type analysis, as described by Trucco 
and de Petris (19). The cell is treated as a molecule with valency, n, 
and n is determined from the x intercept. The y axis of the Scatchard 
plot has the units M^(-1), because the dimensionless ratio, r/(A-Xa), is 
multiplied by 6.02 × 10^20 cells/mmol and divided by the cell concen-
tration in cells per milliliter.

RESULTS

Kinetic analysis of target cell lysis by CTL. Thorn 
and Henney (13) proposed that the maximal velocity of 
target cell lysis (V_max) and the concentration of target cells 
(Km) required for 0.5 V_max could be determined from a 
\(^{51}\)Cr-release assay in which 1) the percent \(^{51}\)Cr release was 
linear with time, and 2) the concentration of target 
cells was varied. In this study the percent \(^{51}\)Cr release 
was linear with time for the first 90 min of the 37°C 
icubation, except for a 21-min lag for the EL4 ascites 
target cells and a 12-min lag for the EL4 t.c. target cells, 
at an effector to target ratio of 20:1 (Fig. 1). A similar lag 
time (15 min) was observed when an assay involving 
preformation of conjugates was used (13).

To determine V_max and Km values, the concentration of target cells was varied from 2.5 × 10^4 to 4.0 × 10^5 
cells/ml, maintaining the anti-H-2K^b T cells constant at 
5 × 10^5 cells/ml. The 37°C assay in suspension was done 
for 90 min, and the percent \(^{51}\)Cr release was used to calculate the velocity of target cell lysis (v). Figure 2 
shows a representative result of v as a function of target 
cell concentration. The two curves level off at different 
values of v, indicating at least a twofold difference in 

\[ v = \frac{(\text{Km} \times \text{target cell concentration})}{(\text{target cell concentration} + \text{V}_\text{max})} \]

V_max. A linear transformation of the data in Figure 2 was 
achieved by plotting v as a function of [v/target cell 
concentration] in an Eadie-Hofstee plot (Fig. 3), according 
to the equation:

\[ v = \frac{(\text{Km} \times \text{target cell concentration})}{(\text{target cell concentration} + \text{V}_\text{max})} \]

The V_max values for EL4 ascites and EL4 t.c. cells, deter-
mined from the y intercepts in Figure 3, were 6.46 × 10^4 
cells/hr and 2.69 × 10^4 cells/hr, respectively, and are 
shown as the end points in Figure 2. The Km values for 
EL4 ascites and EL4 t.c. cells, calculated from the slopes 
in Figure 3, were 1.14 × 10^5 cells/ml and 0.86 × 10^5 
cells/ml, respectively. The V_max and Km values varied 
somewhat from experiment to experiment (Table 1), so the 
values of the ratios of V_max and of Km for EL4 ascites 
to EL4 t.c. cells were used for comparison (Table 1). The 
V_max of EL4 ascites was 2.3-fold higher than that of EL4 
t.c. (the error was 9% in four experiments). The Km ratio 
values were not as reproducible as the V_max ratio values 
(the error was 35% in four experiments), probably be-
cause it is difficult to detect appreciable 51Cr release at target cell concentrations below the Km. Therefore, a CTL-target cell conjugate assay was performed to assess the binding capacity of the differently cultured EL4 cells to the anti-H-2Kb T cells.

**CTL-target cell conjugation.** The anti-H-2Kb T cells and fluorescein diacetate-labeled target cells were mixed at an effector to target cell ratio of 1:1, and conjugation was promoted by centrifugation at 300 X G for 10 min at room temperature. The percent conjugation for EL4 ascites was higher than that for EL4 t.c. (Table II), indicating that the T cells bound better to the ascites than to the t.c. line. This was also observed at effector to target cell ratios of 1:5 and 5:1 (data not shown). The conjugation was shown to be specific because the BW5147.3 target cell (H-2b) did not form as many conjugates as either one of the EL4 target cell lines (Table II).

**Membrane fluidity determination.** A (12,3)-fatty acid spin label was used to determine if there was a difference in membrane fluidity that could explain this difference in reactivity of the target cells toward the anti-H-2Kb CTL. This label was chosen with the spin label near the outside of the membrane because an analogous (10,3)-fatty acid spin label has been used to detect changes in membrane fluidity induced by prostaglandins (20) and cyclic nucleotides (21). The same (12,3)-fatty acid spin label was used to correlate lipid fluidity changes with temperature-induced changes in membrane-bound enzyme activities in lipid-modified Chang liver cell membranes (22).

The spin label was incorporated into the PM fractions from EL4 ascites and EL4 t.c. cells, and the EPR spectra were recorded from 15°C to 40°C, in 5°C increments. In contrast to the study involving Chang liver cell membranes (22), the EPR signal was stable. The EPR spectra at 15.1°C and 40.3°C for EL4 ascites PM fraction are shown as representative examples (Fig. 4). Similar spectra were obtained for EL4 t.c. PM fraction (data not shown). The 2Amax and 2Amin splitting constants were determined from each spectrum, as shown in Figure 4, and the (Amax - Amin) was calculated. The (Amax - Amin) parameter is directly proportional to the membrane order parameter (S), by the formula (23):

\[
S_{\text{approx}} = \frac{(A_{\text{max}} - A_{\text{min}})}{A_{\text{zz}} - 0.5 (A_{\text{xx}} + A_{\text{yy}})}
\]

in which Azz, Axx, and Ayy are the splitting constant values of a single crystal of spin label, when the magnetic field is parallel to the z, x, and y axis, respectively (23). The term \((A_{\text{max}} - A_{\text{min}})\) was plotted against the reciprocal of the absolute temperature (Fig. 5). This plot was similar for the differently cultured EL4 lines, indicating that both PM fractions had similar membrane fluidity in the part of the membrane sensitive to the probe.

**CSP analysis.** Another possibility is that the CSP may vary in the differently cultured EL4 cells, thereby resulting in differential sensitivity toward the CTL. Addition of the CSP to purified H-2Kb in synthetic membranes facilitates the generation of specific H-2 activity (24). Addition of CSP also aids in the incorporation of H-2 into vesicles (S. D. Figard and P. J. Gilmer, manuscript in preparation).

The insoluble material remaining after Nonidet P-40 treatment of the PM fraction is the CSP (17). Volumes of PM fractions from the differently cultured EL4 cells containing equal amounts of membrane protein were solubilized with Nonidet P-40. The insoluble material was prepared for isoelectric focusing and was analyzed by 2D gel electrophoresis (Fig. 6A and B). Most of the proteins were common to both lines; however, the CSP from EL4 t.c. reproducibly exhibited proteins of m.w. 86,500, 70,000, 65,000, and 53,300 (Fig. 6B) that were not present in the EL4 ascites line. A set of the CSP at 35,500 m.w. were present in the ascites (Fig. 6A), but not in the t.c. line. This result shows that there were some differences between the CSP derived from the PM fraction of the differently cultured target cells.

**Comparison of H-2Kb density on EL4 ascites and EL4 t.c. cells.** The H-2Kb antigenic density on EL4 ascites and EL4 t.c. cells was determined by direct binding of 125I-labeled anti-H-2Kb (B8-24-3) mAb (19). Serial dilutions of iodinated mAb were incubated on ice with EL4 ascites, EL4 t.c., BW5147.3, or PBS-0.05% gelatin. The iodinated antibody bound poorly to the negative control BW5147.3 cells, in comparison with either of the EL4 lines (data not shown). The iodinated antibody was experimentally determined to be 72% biologically active after radiolodination (data not shown), according to the method of Trucco and de Petris (19). The cpm bound specifically to the cell sediment (Xa) was obtained by subtracting the nonspecific cpm bound (in tubes containing only PBS-0.05% gelatin) from the observed cpm bound. The value of r, the mAb molecules bound per cell, was calculated by dividing the calculated mAb molecules bound per milliliter by the cells per milliliter. The difference between the calculated cpm input (A) and the specific cpm bound (Xa) yields the cpm due to free mAb molecules. The relationship between r/(A-Xa) and r is linear, as shown in the equation: r/(A-Xa) = (-Kajr + Kaj). The resulting Scatchard plot for the binding of 125I-labeled anti-H-2Kb mAb to EL4 ascites and to EL4 t.c.
Figure 4. Temperature dependence of two EPR spectra at A. 15.1°C and B. 40.3°C of the (12:3)-fatty acid spin label incorporated into the PM fraction of EL4 ascites cells. Essentially the same spectra were obtained for the EL4 t.c. PM fraction. The $2A_{\text{max}}$ and $2A_{\text{min}}$ parameters are indicated. Spectra were obtained under the following conditions: microwave power = 10 mW; field set = 3265 G; receiver gain = $6.3 \times 10^6$; modulation amplitude = 1 x 10$^6$ G; scan range = 100 G; scan time = 4 min; time constant = 0.3 sec; modulation frequency = 100 kHz; microwave frequency = 9.182 GHz. Enlargements shown were performed under the same conditions mentioned, except receiver gain = $6.3 \times 10^6$, scan time = 1 hr, and time constant = 3.0 sec. Spin label was at 2.9% (mol/mol) of total lipid (see Materials and Methods).

Figure 5. Plot of $(A_{\text{max}} - A_{\text{min}})$ in gauss (G) as a function of $1/10^3$ for EL4 ascites (C) and EL4 t.c. (D) PM fractions. $A_{\text{max}}$ and $A_{\text{min}}$ parameters were determined from the EPR spectra of (12:3)-fatty acid spin label as shown in Figure 4. The best straight line was determined by linear regression analysis.

cells (Fig. 7) was used to determine the affinity constant, $K_a$, and the minimum number of anti-H-2K$^b$ binding sites per cell, n (Table III). This value, n, is the number of binding sites per cell, assuming that the mcAb binds primarily monovalently, under the conditions tested (see Discussion). If the mcAb were binding totally bivalently, then the actual number of sites would be twice the value of n. The values determined for the number of H-2K$^b$ molecules per cell (Table III) agree fairly well with 2 to 3 x 10$^5$ total H-2 sites per normal lymphocyte (25), 2 to 5 x 10$^6$ per mouse lymphoblast (26), 0.5 to 1.1 x 10$^5$ per spleen cell (27), 0.6 to 1.5 x 10$^5$ per lymphocytic leukemia cell (10), and 0.4 to 2.1 x 10$^5$ sites/cell for BW5147 thymoma cells (25). The ratio of $K_a$ values for EL4 ascites to that for EL4 t.c. was 1.4 (Table III), indicating a similar affinity constant of $^{125}$I-iodinated anti-H-2K$^b$ mcAb for the H-2K$^b$ antigen on the two cell surfaces. The ratio of n for EL4 ascites to that for EL4 t.c. was 2.7, however, indicating 2.7-fold more anti-H-2K$^b$ mcAb binding sites were present on EL4 ascites (Table III).

DISCUSSION

We previously demonstrated that EL4 target cells harvested from ascites are significantly better target cells for allogeneically primed CTL than when they are grown in t.c. (9). One difference between the differently cultured target cells is the amount of neuraminidase-releasable sialic acid, with the more susceptible cell exhibiting less sialic acid (9). In the study presented here, the antigenic density of H-2K$^b$ molecules, the membrane fluidity, and the CSP present in the differently cultured target cells lines were examined in an effort to explain the difference in susceptibility to attack by CTL.

Because the CTL are directed primarily against the H-2K$^b$ antigens (10), the H-2K$^b$ antigenic density on the differently cultured target cells was determined by using radioiodinated anti-H-2K$^b$ mcAb. The Scatchard-type analysis in Figure 7 showed a linear dependence with a negative slope, as expected. The conditions for the Scatchard plot were chosen so that monovalent binding of the mcAb would predominate. This predominance was achieved by maintaining the number of active mcAb molecules (150,000 m.w.) in excess of the total number of sites on the cells. For instance, in Figure 7, the total number of H-2K$^b$ sites was $1.0 \times 10^{11}$ and $3.8 \times 10^{10}$ for EL4 ascites and EL4 t.c., respectively, and the number of active mcAb molecules (150,000 m.w.) in the most concentrated tube was $18.7 \times 10^{11}$. The Scatchard plot deviated from linearity once the mcAb was diluted lower than the number of sites (data not shown). We conclude from the data of Mason and Williams (28) that the rate constant for dissociation of univalently bound B8-24-3 must be small.
It is unlikely that the mcAb bound monovalently to one cell surface and bivalently to the other, because the binding affinity for the radioliodated mcAb was similar for both cell types (Table III). Assuming this 1:1 stoichiometry for the binding of mcAb to the cell surfaces, the value of $n$ equals the number of H-2K$^b$ molecules on each cell. Therefore, there were 2.7-fold more H-2K$^b$ antigens on the more susceptible EL4 ascites than the less susceptible EL4 t.c. target cell (Fig. 7, Table III). Similarly, Hackett and Askonas (25) observed a fivefold increase in total H-2K$^b$ expression of BW5147 thymoma cells when the cells were grown in ascites rather than in t.c., but the antigenic density of the Thy-1.1 glycoprotein is unaffected.

The H-2K$^b$ antigenic density may be affected by differences in either the phase of growth of the cells when isolated (29), the level of sialic acid present on the H-2K$^b$, the turnover of antigens, or some other variable. Both target cell lines were isolated in the log phase of growth, so the first alternative is unlikely. The second possibility was tested by treating the cells with neuraminidase (V. cholerae) to determine if neuraminidase treatment could augment the number of sites. EL4 t.c. was treated with neuraminidase, as described (9), for 80 min at pH 7.0, and then was allowed to recover for 50 min in glucose-containing medium. There was no significant increase in the number of H-2K$^b$ sites or, in contrast to another study (26), in the affinity constant for this treatment (data not shown). Longer recovery was not possible due to loss in cell viability. Also, Buck and Warren (30) found no stimulation of isotopic incorporation into various cell surface components upon neuraminidase treatment. The third alternative is possible because the turnover and the shedding of the antigens may be affected by the cytoskeletal structure, which we have shown is different (Fig. 6A and B). Currently, we are testing whether there is a difference in the rate of shedding of antigens or if some other variable, such as the pH in which the cells were grown, affects the number of H-2K$^b$ sites per cell.

The T cell-mediated lysis of a target cell is composed of at least three steps (31, 32): 1) binding of the target cell to the T cell; 2) programming for lysis that culminates in lysis of the target cell; and 3) recycling of the T cell. The rate-limiting step is unknown and may even depend on the assay conditions employed (reviewed in Reference 2). At the molecular level, however, the parameters that control the lytic reaction are poorly understood.

In an effort to compare the binding and the maximal velocity of lysis of the differently cultured target cells by the T cells, the velocity of lysis, under conditions of steady-state release of $^{51}$Cr, was analyzed by analogy with Michaelis-Menten kinetics (13). This analysis assumes that 1) a steady-state release of $^{51}$Cr is achieved (Fig. 1); 2) the binding of the T cell to the target cell is fast (31) and reversible (33); and 3) the rate-determining step is $^{51}$Cr release from the target cells. Assuming that the lytic reaction does fit the assumptions for Michaelis-Menten kinetics, a 2.3-fold increase in calculated $V_{\text{max}}$ in the $^{51}$Cr release assay was observed for the EL4 ascites target cell in comparison to the EL4 t.c. cell (Table I).

The 2.3-fold increase in $V_{\text{max}}$ may be a direct result of the 2.7-fold increase in H-2K$^b$ antigenic density observed for the EL4 ascites cells. The idea that the antigenic density difference may cause the difference in $V_{\text{max}}$ is consistent with the model for CTL-target cell interaction, as suggested by Berke and Clark (34). This conclusion is
The ratio of \( K_m \) values determined from the kinetic experiments did not offer a reproducible means of comparing the binding of the differently cultured target cells to the anti-H-2K\(^b\) CTL (Table I), so binding was assessed more reproducibly by conjugate experiments (Table II). The percent T cells bound to target cells was significantly greater with the more susceptible EL4 ascites than with the EL4 t.c. target cell (Table II). Presumably, this difference in binding to T cells may be due to the difference in either the H-2K\(^b\) antigenic density on the target cells, the CSP, or some other unmeasured biochemical property. Therefore, the lower amount of lysis of EL4 t.c. cells (Fig. 2. Reference 9) was apparently due both to a decreased binding of target cells to T cells and to a decreased \( V_{max} \). Only a small difference in conjugate formation was reported previously with the use of the same T cells with the two differently cultured target cells (9), although in those experiments conjugates were determined by the percent targets bound to T cells at an effector to target ratio of 5:1. Apparently, the system is more sensitive to detecting differences in binding when conjugates are scored as the percent T cells bound to targets (15).

Two other factors that were investigated in the differently cultured target cell lines included membrane fluidity and the CSP. Although the transmembrane (35) MHC antigens have a dispersed distribution and are mobile in the plane of the membrane (36), they also interact with actin, one of the CSP (37–39), possibly via a phosphorylation site on the cytoplasmic portion of the transmembrane antigen (40–42). Therefore, changes in the membrane fluidity or in the cytoskeleton could influence the lateral mobility of the H-2 proteins, thereby influencing the target cell binding to the CTL and/or the lytic process.

Membrane fluidity was examined by using a spin label probe. There was no change in the (\( A_{max} - A_{min} \)) parameter, which is proportional to the membrane order parameter, for the differently cultured target cells (Fig. 5). Results of other studies (22) suggest it is possible that even though we did not observe any difference in fluidity between the PM of the differently cultured cells, some difference may actually exist.

The CSP from the PM of the differently cultured target cells were isolated and compared by 2D gel electrophoresis. Basically, some differences were observed (Fig. 6A and B); however, 2D gel analyses of in vitro \(^{35}S\)methionine-labeled PM preparations are essentially identical from EL4 ascites (11) and EL4 t.c. (10). Many of the silver-stained CSP were not observed on the methionine-labeled PM gels, the most likely reason being that CSP were not appreciably labeled with methionine in the 90-min labeling period.

In summary, the difference in susceptibility to lysis of the differently cultured EL4 target cells by anti-H-2K\(^b\) CTL was due to either 2.7-fold more H-2K\(^b\) sites on the EL4 ascites than the EL4 t.c. cell (Table III); the difference in CSP present in the two cells (Fig. 6A and B), or some other biochemical property, as yet unmeasured. The more susceptible ascites target cells exhibited a 2.3-fold higher \( V_{max} \) (Table I) and increased binding to the anti-H-2K\(^b\) T cells (Table II). There was no detectable difference in fluidity between the differently cultured target cells (Fig. 5).

Acknowledgments. We thank Profs. William Marzluff

![Figure 7](https://www.jimmunol.org/)

Figure 7. Scatchard analysis of the specific binding of \(^{125}I\)-labeled anti-H-2K\(^b\) (B6-24-3) mAb to EL4 ascites (•) or EL4 t.c. (○) cells. Each determination was performed in duplicate; standard deviation is shown. The best straight line was determined by linear regression analysis. The x intercept of the plot is equal to the number of binding sites (n) of 150,000 m.w. antibody per cell, assuming that the mAb bound monovalently (see text). The slope is equal to the negative of the affinity constant (K\(a\)), and the y intercept is equal to (K\(a\)n). The values of K\(a\) and n are given in Table III. A separate experiment showed that the radiolabeled mAb was 72% active (data not shown).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Target Cell</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_a \times 10^{-6}, \text{M}^{-1} )</td>
<td>EL4 ascites</td>
<td>EL4 t.c.</td>
</tr>
<tr>
<td>( n \times 10^{-9} )</td>
<td>13 ± 1</td>
<td>9.5 ± 0.7</td>
</tr>
</tbody>
</table>

* Values of K\(a\) and n were calculated from the slopes and x intercepts, respectively, of the plots of r/(A-Xa) vs r, as in Figure 7. Radiolabeled mAb was 72% active. Values of n were calculated assuming that the mAb bound monovalently (see Discussion).

* The EL4 target cells were grown in ascites or in t.c.

* Ratio refers to the parameter for EL4 ascites vs the parameter for EL4 t.c.

also consistent with the results of Kuppers et al. (5) in which a decrease in T cell-mediated lysis occurs in parallel with the loss of papain-cleaved, serologically detectable H-2 antigens on the target cell surface, and 2) in H-2 variant cell lines displaying decreasing amounts of H-2 products. The antigenic density cannot be the absolute controlling property, however, because in other studies a stialic acid-deficient variant cell is lysed no better than the parent EL4 t.c. cell, despite a 2.5-fold higher H-2K\(^b\) antigenic density on the variant cell (10). It is also possible that the differences in the CSP or some other untested biochemical property of the cells may have caused the difference in \( V_{max} \).
and Kenneth Roux for helpful discussions during the course of this study, and Ms. Melani Sullivan for writing the computer program for analyzing the data from the mAb direct binding experiments.

REFERENCES


