Inhibition of Specific Effector T Cell-Target Cell Conjugates by Target Cell Plasma Membranes

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INHIBITION OF SPECIFIC EFFECTOR T CELL-TARGET CELL CONJUGATES BY TARGET CELL PLASMA MEMBRANES

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Conjugate formation between effector T cells raised in vivo in a BALB/c host and fluorescein diacetate-labeled allogeneic EL4 tumor target cells was observed and scored by using a fluorescence microscope. This conjugate formation was found to be specifically inhibited by plasma membrane vesicles isolated from the EL4 cells.

Partially reconstituted stimulator and target cell membranes having defined chemical compositions and physical properties are of potential interest for studies of cell-cell recognition, cell-cell stimulation, and effector cell-target cell killing, provided these reconstituted membranes can be shown to retain the properties of their in vivo and in vitro cellular counterparts. We report here evidence for the specific inhibition of the formation of conjugates between BALB/c anti-EL4 effector T cells and EL4 tumor target cells by plasma membrane vesicles isolated from the EL4 cells. The BALB/c effector cells were prepared by nylon wool purification of peritoneal exudate cells from BALB/c mice after injection of γ-irradiated EL4 cells into the peritoneal cavities of these mice. The present studies provide support for the view that partially reconstituted target membranes may have the capacity to mimic in vitro the target specific properties of intact cellular plasma membranes.

MATERIALS AND METHODS

Mice. BALB/c female (H-2b) mice used in this study were obtained from our breeding colony, and the C57BL/Ka (H-2b) female mice were kindly provided by Dr. Miriam Lieberman, Department of Radiology. C57BL/6J female mice from Jackson Laboratories were used to maintain the EL4 cells. Animals were 2 to 3 months old when used.

Cell lines. EL4 (H-2b) is a lymphocytic leukemia line derived from C57BL/6, and S1A.4 (H-2b) is a lymphocytic lymphoma derived from BALB/c female mice. Both lines are available from the Salk Institute.

S1A.4 was grown in suspension in Dulbecco's modified Eagle's medium (GIBCO No. 188G, Grand Island, N. Y.) plus 5% heat-inactivated fetal calf serum (FCS) plus penicillin streptomycin in a humidified 5% CO2 incubator at 37°C, and was harvested after 3 days at a final cell density no greater than 10^6 cells/ml. Cell viability typically was 90 to 95%. The EL4 cells were maintained in vivo in the peritoneal cavities of C57BL/Ka female or C57BL/6J female mice by weekly transfer of 5 x 10^6 live cells per 0.5 ml injection in phosphate-buffered saline (PBS). Typically, 10^6 EL4 cells were harvested per mouse after a 6 to 7 day growth of the tumor cells. Cell viability was greater than 95%.

Membrane preparations. Single cell suspensions from spleens were prepared by gently shearing each spleen between two frosted microscope slides and suspending the cells in PBS-5% FCS (PBS/FCS). In a typical spleen cell plasma membrane preparation, 40 to 50 spleens were used. Tissue debris was removed by filtering the suspension through nylon net (mesh 86 μ). Red blood cells were lysed by treating with 1.6 ml 0.75% NH4Cl, 17.0 mM Tris, pH 7.2, per spleen for 10 min at room temperature and the remaining cells were washed twice in PBS/FCS.

Preparations enriched for plasma membranes were made from murine tumor lymphocyte lines or from normal murine spleen cells. The cells were swollen by incubation of a 1% (by volume) cell suspension in reticulocyte standard buffer (10 mM Tris-HCl, 10 mM NaCl, 1.5 mM MgCl2 and, 0.2 mM CaCl2, pH 7.40) for 15 min (for tumor cells) or 5 min (for spleen cells). The plasma membrane was broken by using nitrogen cavitation. At least 80% of the cells were broken and most nuclei remained spherical and intact. For the purification of the plasma membrane fraction we employed the protocol of Crumpton and Snary (1). In this method three membrane fractions are obtained in a sucrose step density gradient. Only the interface fraction (predominantly plasma membrane vesicles) was used in the experiments discussed here. Further characterization of this fraction and the pellet fraction (largely endoplasmic reticulum) will be given elsewhere (Gilmer, to be published). Aliquots of membrane preparations were stored frozen in vials in 31% sucrose-10 mM Tris (pH 7.5) after pooling of the interface fractions. Plasma membranes are stable to recognition by specific antibody under these conditions. BALB/k (H-2k) membrane vesicles used in the present work as controls were kindly supplied by Dr. C. Furlong and were prepared by the same method.

Specific antibodies against H-2Dk were raised against B10.A(2R) lymphocytes in [A × B10.A(5R)]F1 mice by D. Murphy. Likewise, antibodies against H-2Kk were raised against B10.A lymphocytes in [A.TL × B10.A(5R)]F1 mice.

The binding of antibodies specific for H-2Dk and H-2Kk antigens by EL4 cells and ELA membrane vesicles was determined by the C microcytotoxicity assay described by Murphy and Shreffler (2) with CSW lymph node target cells with the H-2Dk antisera, and C3H lymph node target cells with the H-2Kk antisera. Data are given in Figure 1. These data demonstrate the specificity of antibody binding to the plasma membrane vesicles. The displacement of the curves in Figure 1A
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Figure 1. Test of antibody-dependent, rabbit complement-mediated cytotoxicity of antisera absorbed against intact EL4 cells (○—○) or plasma membrane preparation from EL4 cells (□—□) (see text).

<table>
<thead>
<tr>
<th>Target</th>
<th>Antiserum</th>
<th>Dilution of Antiserum</th>
<th>Volume of Antiserum Absorbed</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. CSW (H-2k)</td>
<td>Anti-D&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1/320</td>
<td>80 µl</td>
</tr>
<tr>
<td>B. C3H (H-2&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Anti-K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1/160</td>
<td>80 µl</td>
</tr>
</tbody>
</table>

indicates a yield of about 30% membranes from EL4 cells (assuming all vesicles are closed, and right side out).

Cells (or plasma membrane cell equivalents) were serially diluted and then the appropriate concentration of antiserum was added for the absorption and incubated for 60 min with occasional mixing at room temperature. The cells were centrifuged at 250 × G and the membranes at 90,000 × G. The absorbed sera were frozen at −80°C and later tested for cytotoxicity. All absorbed serum samples were tested in duplicate in the microcytotoxicity test with appropriate controls including normal mouse serum, normal mouse serum plus C, and C alone.

Inhibition of conjugate formation by plasma membrane vesicles. Effector T cells were raised in vivo in a BALB/c male host (H-2<sup>+</sup>) by a modification of the method of Berke against allogeneic tumor cells (EL4; H-2<sup>+</sup>) (S. Hurt, G. Berke, and W. Clark, to be published). Typically, 50 × 10<sup>6</sup> irradiated (2000 rads) EL4 cells were injected i.p., and the peritoneal cells were collected 4 to 5 days later. A T cell effector population was prepared by incubating these peritoneal cells in PBS/FCS on a nylon wool column for 45 min at 37°C (3). Control cells were raised either in C57BL/Ka mice against EL4 targets or in BALB/c mice against thioglycollate (4). The nylon wool purified effector or control cells were suspended at 3 to 5 × 10<sup>6</sup> cells/ml in PBS/FCS and used at this concentration. Target EL4 cells were obtained fresh from the peritoneal cavities of syngeneic C57BL/Ka mice. T-effector cells showed fully cytotoxic activity against Cr<sup>51</sup>-labeled EL4 targets.

The target cells were loaded with fluorescein diacetate (FDA)<sup>3</sup> by diluting a 0.01% FDA solution (containing 2% ace-

<sup>3</sup> Abbreviations used in this paper: FDA, fluorescein diacetate; TBS, 10 mM Tris, 150 mM NaCl, pH 7.4.

Effects and targets were mixed at a 5:1 ratio in a total volume of 25 µl in Cooke pro-vials, and 10 µl of serial dilutions of Dounced homogenized plasma membrane preparations in 10 mM Tris, 150 mM NaCl, pH 7.4 (TBS) were added. The vials were centrifuged at 1325 × G for 15 min in the cold and left on ice until conjugates were counted in the microscope. Conjugates were easily detected because the target cell is large and fluoresces strongly, and the effector is small and does not fluoresce, or fluoresces weakly (see below). We used a Zeiss Photomicroscope equipped with a mercury lamp with epi-fluorescence and Nemarsky optics.

The conjugates were scored as the percentage of targets binding at least one effector. A field was found containing between 10 to 40 FDA-loaded targets by using the mercury lamp only. Then the lamp source was switched manually from fluorescence to normal illumination, and each target was scored for presence of a bound effector. Samples were observed in duplicate. It is common to see more than one effector bound to a single target. Also sometimes one effector may bridge two targets, and in this case both targets are scored as positive for presence of a bound effector.

FDA-loaded peritoneum-derived target tumor cells alone showed virtually no small lymphocyte contamination that might otherwise be confused with effector cells.

RESULTS AND DISCUSSION

Figure 2 gives histograms for the percent of target EL4 cells that were found to be conjugated with one or more effector cells, in comparison with the number of conjugates formed in the presence of several concentrations of (inhibitory) target plasma membranes derived from the EL4 cells. Controls include BALB/c (H-2<sup>+</sup>) target cell controls (SIA4); nonimmune peritoneal cell controls ("BALB/c anti-thioglycollate"); and spleen vesicle membranes from a third party strain, BALB/k (H-2<sup>+</sup>). In the present study ~57 ± 10% of the EL4 targets were bound to one or more BALB/c anti-EL4 effectors, whereas only ~19 ± 10% of SIA4 targets were conjugated to BALB/c anti-EL4 effectors, and 28 ± 10% of the EL4 targets were bound to nonspecific BALB/c effectors ("anti-thioglycollate"). In other similar experiments the specific conjugates were somewhat higher, ~70%, and control values for effector cells raised in syngeneic host were of the order of 30% (C57BL/Ka anti-EL4). In all these experiments targets bearing one or more effectors were scored as singles.

The data in Figure 2 provide evidence for the inhibition of specific effector T cell-target cell conjugates by target cell plasma membrane vesicles. Our use of high concentrations was prompted by the studies of Ryser et al. (6) who used high concentrations of effector cells and target cells for efficient isolation of specific allogeneic conjugates.

In the present study we have made (and recorded photographically) the following additional observations. a. When membrane vesicles prepared from specific target cell plasma membranes are loaded with isotonic carboxyfluorescein (78 mM, pH 7.2) these vesicles can sometimes be observed to be bound to effector cells. Under experimental conditions compa-
effect of the vesicles then requires that many more vesicles be bound but not observed (say, through loss of carboxyfluorescein) or that these vesicles have in some other fashion inhibited the effector cells. In this connection, see Elliott et al. (7), and also Weinstein et al. (8).

b. A number of effector cells, some bound to targets and some free of targets, are seen to fluoresce weakly, evidently due to the presence of fluorescein. A large proportion of these effector cells (e.g., 50%) do not fluoresce detectably; often when an effector cell is found to fluoresce it is bound to a target cell, and sometimes this conjugated target cell is observed to have a reduced fluorescence intensity. This then provides evidence for transfer of fluorescein between target and effector cells (both specific and nonspecific pairs). These results appear to be in accord with those of Sellin et al. (5, 9) and not in accord with those of Kalina and Berke (10).

On the basis of the present study, we anticipate that reconstituted membranes of defined composition will be capable of specifically inhibiting effector cell-target cell conjugate formation and should shed light on the chemical and physical properties required for this inhibition.

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REFERENCES


