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Membrane-Reconstituted, Purified H-2K^b Specifically Inhibits T-Cell–Target Cell Conjugate Formation[1]

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We report the use of a sensitive microassay to detect purified H-2K^b antigens which have been functionally reconstituted into membrane vesicles of defined composition. The histocompatibility antigens have been purified by monoclonal antibody affinity chromatography. The assay utilizes inhibition of specific conjugate formation between allogeneically primed (*H-2^d* anti- *H-2^b*) cytotoxic T cells and *H-2^b* target cells by the membrane-reconstituted H-2K^b antigens. Cytoskeletal proteins were added to the H-2K^b (and control H-2^k) antigens. Sucrose density fractionation of reconstituted vesicles and Pronase E cleavage studies suggested that the cytoskeletal proteins aided in the incorporation and vectorial orientation of the antigens into large, cholesterol-containing membrane vesicles. As little as 6 ng purified H-2K^b plus 28 ng cytoskeletal proteins in vesicles of defined lipid composition (0.28, 0.25, 0.47 mol fraction cholesterol, dimyristoylphosphatidylcholine, and dipalmitoylphosphatidylcholine, respectively) inhibited specific conjugate formation to 50% of the maximum inhibition observed. This inhibition was shown to be specific in two ways: (i) the same H-2K^b-containing vesicles did not inhibit nonspecific conjugate formation, and (ii) control vesicles containing the same amounts of lipid, cytoskeletal proteins, and purified H-2^k proteins inhibited conjugate formation but only at significantly higher H-2^k concentrations, indicating the specificity of the response with the vesicles containing H-2K^b. © 1985 Academic Press, Inc.

INTRODUCTION

Model membranes of defined lipid composition containing defined amounts of antigens or haptens have been used elegantly to study the factors which influence the humoral branch of the immune system (1). Recently, analogous studies involving the use of model membranes to study the T-cell branch of the immune system have been reported using either purified plasma membrane proteins or purified histocompatibility antigens as the stimulator or the target for T cells.

The most utilized assay system involves the stimulation of a secondary cytotoxic-T-cell response *in vitro* using H-2 or HLA antigens reconstituted into lipid vesicles. Membrane-reconstituted proteins or the isolated plasma membrane proteins were used to restimulate spleen cells which had been primed *in vivo* either allogeneically

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or syngeneically to a viral protein (2–10). Immune specificity was preserved using these model membrane systems. However, since at least either the proteins or the lipids were not pure, it was not possible to characterize fully all membrane parameters which are critical for biological activity.

As it became possible to isolate purified HLA or H-2 antigens, the pure proteins were incorporated into vesicles, but cell membrane lipids instead of purified lipids were used in the reconstitution (11–13). Purified H-2^k antigens were found to be more efficient stimulators (13) when the membrane reconstitution was performed in the presence of the detergent-insoluble, cytoskeletal proteins (CP)[3] (14).

The other method used to test the functional activity of histocompatibility antigens is an assay involving inhibition of conjugate formation between specific cytotoxic T cells (CTL) and target cells (TC) by membrane-incorporated or detergent-solubilized major histocompatibility antigens (15–17). Two major advantages to this conjugate assay method are that the assay is short term and that small amounts of reconstituted material are required. This method was chosen to test the biologically functional activity of purified histocompatibility antigens reconstituted into membrane vesicles of defined lipid composition. The pure lipids were the same as those which had been utilized to form membrane vesicles used as specific synthetic targets for allogeneically primed CTL (18). The CP were added with the purified antigens in the reconstitution. Immune specificity was demonstrated (i) by observing inhibition of conjugate formation by H-2K^b only with specific TC but not with third party TC and (ii) by using control vesicles containing H-2^k.

MATERIALS AND METHODS

Mice. BALB/c and C57BL/6 mice were obtained from either Charles River Laboratory, Wilmington, Massachusetts or our own breeding colony.

Cell lines. The following cell lines are available from either the Salk Institute, La Jolla, California or the American Type Culture Collection, Rockville, Maryland: EL4 (*H-2^b*), a lymphocytic leukemia murine line derived from C57BL/6; BW5147.3 (*H-2^k*), a lymphoma line from a spontaneous AKR/J tumor; M1/42.3.9.8 (M1/42), a hybridoma line obtained from a DA rat anti-C57BL/10 mouse fused with NS1; and a murine hybridoma B8-24-3 (anti-H-2K^b). The EL4 cells were maintained in ascites form in the syngeneic C57BL/6 female by passage of 5×10^6 cells intraperitoneally every 5–7 days or in tissue culture (tc) by passage every 2–3 days at 37°C in a 5% CO_2 incubator in Dulbecco's modified Eagle's medium (DMEM, GIBCO No. 430-1600, Grand Island, N.Y.) containing 10% heat-inactivated horse serum (HS, GIBCO) and 1% penicillin-streptomycin (PS, Eli Lilly, Indianapolis, Ind.). The BW5147.3 line was grown identically to EL4 tc in spinner flasks. Cell viability typically was >98% for EL4 ascites and >90% for BW5147.3 and EL4 tc.

Monoclonal M1/42 antibody purification and coupling to Sepharose-4B. M1/42 hybridoma secretes monoclonal antibody (mAb) which recognizes H-2 antigens of

[3] Abbreviations used: CP, cytoskeletal proteins; CTL, cytotoxic T lymphocytes, DMEM, Dulbecco's modified Eagle's medium; EGTA, ethylene glycol bis(β-aminoethyl ether) *N,N'*-tetraacetic acid; FCS, heat-inactivated fetal calf serum; HS, heat-inactivated horse serum; mAb, monoclonal antibody; M1/42, M1/42.3.9.8 hybridoma; PBS, phosphate-buffered saline (2.7 m*M* KCl, 1.5 m*M* dihydrogen potassium phosphate, 0.49 m*M* magnesium chloride, 137 m*M* NaCl, 0.81 m*M* disodium phosphate, 0.9 m*M* calcium chloride); PS, penicillin–streptomycin; tc, tissue culture; TC, target cell.

many haplotypes, including H-2^k (19). The hybridoma M1/42 cells were maintained at high viability by growth in DMEM containing 5% heat-inactivated fetal calf serum (FCS, GIBCO) and 1% PS. When antibody was needed, the cells were washed free of the serum and then grown for 3–4 days in the following serum-free medium: DMEM containing 20 μg/ml human transferrin (Calbiochem–Behring, San Diego, Calif.), 10 μg/ml bovine insulin (Sigma, St. Louis, Mo.), 20 μM ethanolamine (Sigma), 0.6 mg/ml L-glutamine (Sigma), 17.3 ng/ml Na selenite (Aldrich, Milwaukee, Wisc.), 5 mM LiCl (Baker, Phillipsburg, N.J.), 1% nonessential amino acids (GIBCO No. 320-1140), and 1% PS. These conditions were modifications of other serum-free media chosen for other cell lines (20–22). Cell density doubled in the serum-free medium. Although cell viability dropped appreciably by the time of harvest, antibody was still secreted into the medium. An enzyme-linked immunosorbent assay was adapted (23) to determine antibody activity using EL4 tc cells in DMEM–10% HS–1% PS and horseradish peroxidase-linked rabbit anti-rat IgG (H + L) (Bionetics, Kensington, M.) (data not shown).

The M1/42 cell-free supernatant was concentrated 100-fold with either an Amicon XM50 or a PM10 filter. The mAb was purified by recrystallization three times from 50% ammonium sulfate (Baker) at room temperature. The M1/42 was coupled at 50 mg CNBr/1.0–2.0 mg antibody/ml Sepharose 4B (Sigma) (24). Lower levels of CNBr activation (25 mg CNBr/ml Sepharose 4B) yielded an affinity column that required harsher conditions to elute the H-2 antigens.

Purification of B8-24-3 mAb. B8-24-3 (anti-H-2K^b) mAb was purified, as described (25).

H-2^k purification. The H-2^k antigens were obtained from BW5147.3 cells using an M1/42-Sepharose 4B affinity column (0.9 × 2.0 cm), as described (26, 27), with some minor modifications. NP-40 detergent (Particle Data Labs, Elmhurst, Ill.) was used instead of Triton X-100, and a low-salt wash (0.5% NP-40, 0.15 M NaCl, 20 mM Tris–HCl, pH 8.0) was included before sample elution. The eluted antigens were diluted twofold to prevent the buffer from gelling, immediately dialyzed versus 0.3% Na deoxycholate, 0.10 M NaCl, 20 mM Tris–HCl, pH 8.0, and concentrated fivefold to 200–500 μl by vacuum dialysis. The antigens were quantitated by the Folin method (28), using bovine serum albumin as the standard, using $A_{280\ nm} = 0.667$ for 1 mg/ml (29). Typically, 0.7–1.0 × 10^9 cells were solubilized, yielding 18 μg H-2^k/10^9 cells.

A sample was radioiodinated to a specific activity of 3.0 × 10^3 cpm/μg protein using the solid state lactoperoxidase method (30). Free [^{125}I]iodide was removed from the radiolabeled H-2 antigens on a Sephadex G-25 medium (Pharmacia Fine Chemicals, Piscataway, N.J.) column (1.6 × 12 cm) at room temperature, equilibrated, and washed with 0.3% Na deoxycholate, 0.1 M NaCl, 20 mM Tris–HCl, pH 8.0. Prior to use in the liposome formulations, the ^{125}I-antigens were concentrated in a Centricon 10 microconcentrator (Amicon, Danvers, Mass.) at 4°C and restored to the original volume, and then the radioactivity was quantitated. This was repeated, typically three to four times, until the radioactivity in the retained protein was constant.

H-2K^b purification. The H-2K^b antigen was isolated from EL4 ascites using the same solubilization procedure, as described above. A B8-24-3-Sepharose 4B affinity column (0.9 × 2.0 cm) was used using a different set of washing and eluting buffers, adapted from Albert *et al.* (31). After the affinity column was loaded with the clarified

cell extract, the column was washed with 50–100 ml 0.25% NP-40, 20 m*M* Tris–HCl, pH 8.0, and then with 50–100 ml 0.25% NP-40, 0.15 *M* NaCl, 1 m*M* EDTA, 20 m*M* Tris–HCl, pH 7.4. The column was washed with 5 ml 1 *M* Tris–HCl, pH 7.8, followed by 5 ml 0.3% Na deoxycholate, 0.10 *M* NaCl, 20 m*M* Tris–HCl, pH 7.8, and finally by 5 ml 1 *M* Tris–HCl, pH 7.8. The antigen was eluted with 5 ml 30 m*M* octyl β-glucoside (Calbiochem–Behring), 0.10 *M* NaCl, 2 *M* ammonium thiocyanate (Baker), 50 m*M* Tris–HCl, pH 7.4. The eluted antigen was immediately applied to a Sephadex G-25 medium (Pharmacia Fine Chemicals) column (1.6 × 12 cm) at room temperature to remove the ammonium thiocyanate and eluted with 0.3% Na deoxycholate, 0.10 *M* NaCl, 20 m*M* Tris–HCl, pH 8.0. Thereafter, the antigens were treated identically to the H-2^k antigens, except that the dilution step to prevent gelling was not necessary. Typically, 1–5 × 10^9 cells were solubilized yielding 12–14 μg H-$2K^b$/10^9 cells. The H-$2K^b$ antigens were ^{125}I-iodinated to a specific activity of 2.0 × 10^4 cpm/μg protein and separated from [^{125}I]iodide, as described above.

One-dimensional gel analysis. The one dimensional gels were performed as described (32).

Isolation of cytoskeletal proteins. The CP were isolated from BW5147.3 cells as described (14), except as noted (25). Additionally, dounce homogenization of the plasma membranes in the presence of NP-40 was found to stabilize the final CP preparation for the membrane reconstitution.

Liposome formation. The lipid composition for the reconstitution was identical to that of Hollander *et al.* (18). The stock solution contained 10 mg dipalmitoylphosphatidylcholine monohydrate, 5 mg dimyristoylphosphatidylcholine monohydrate, 3 mg cholesterol in 3 ml chloroform (Mallinckrodt, Paris, Ky.; spectrophotometric grade) and was stored under N_2 at -70°C. The cholesterol (Sigma) was purified using redistilled dichloromethane (Mallinckrodt) as the solvent on a Sephadex LH-20 (Pharmacia Fine Chemicals) column (33). It was stored under N_2 to prevent oxidation which causes immunosuppression in another immunological system (34). The phospholipids (Supelco, Bellefonte, Pa.) and cholesterol were shown to be pure by thin-layer chromatography on 250μm silica G60 plates using chloroform:methanol:water (65:25:4) and benzene:ethyl acetate (3:2), respectively. The purified cholesterol was quantitated using the Lieberman–Burchard reaction (35).

The vesicles were prepared by first drying a total of 60 μg of lipid as a film twice under N_2 from chloroform. The film was dissolved in a solution containing 25 μg CP plus 5 μg H-$2K^b$ (or 5 μg H-2^k), doped with a small amount of ^{125}I-iodinated H-2, in 0.25 ml 0.2–0.5% Na deoxycholate, 0.15 *M* NaCl, 20m*M* Tris–HCl, pH 8.0, so that the total lipid/total protein ratio was 2/1 (w/w). The resulting solution was dialyzed for 36–48 hr using three 0.5-liter changes of PBS at 4°C. The second PBS buffer also contained 1% PS. The final CP/H-2 ratio in the recovered vesicles, as determined by total protein determination (Folin assay) and by H-2 antigen recovery (recovered radioactivity), varied from 2/1 to 6/1.

Sucrose density-gradient centrifugation. Vesicles were prepared, as described above, except that the preparation also contained tracer [^{14}C]cholesterol (New England Nuclear, Boston, Mass.). To 100 μl of the liposome preparation was added either 100μl PBS or 100 μl 1 mg/ml Pronase E (*Steptomyces griseus*, type XIV, Sigma), dissolved in PBS–0.02% Na azide. It was incubated for 2 hr at room temperature. Solid sucrose (Baker) was added to adjust the sucrose concentration to 55% (w/w) and a 0–50% (w/w) sucrose gradient in PBS–0.02% Na azide was layered on top. The gradients were

centrifuged for 18 hr at 180,000*g* at 4°C. Fractions were collected from the top of the centrifuge tubes and assayed by scintillation and gamma counting. The density was determined using a refractometer.

Inhibition of conjugate formation. The T cells were primed allogeneically by stimulating a BALB/c (*H-2*^{d}) female mouse with 2×10^7 irradiated (2000 rad) EL4 (*H-2*^{b}) cells. The peritoneal cells were harvested 5–6 days later and purified, as described (36). This population of purified T cells contains 30–35% CTL (37, 38). Basically, the same procedure for forming conjugates was used (15), with minor modifications, as indicated. The T cells after purification and the TC after fluorescein diacetate loading were resuspended in Ca^{+2}-free PBS containing 5 m*M* ethylene glycol bis(β-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA) and 5% FCS. Each sample was prepared in 35 μl, and the components were added in the following order: vesicles containing H-2 antigens plus CP were mixed with 3.16×10^4 T cells and incubated at 37°C in a 5% CO_2 incubator for 1 hr. After 10 min at room temperature, 1.58×10^4 fluorescein diacetate labeled TC were added to the mixture, resulting in a T cell/TC ratio of 2/1. After centrifugation for 15 min at 1325*g* using a swinging-bucket rotor at room temperature (27–29°C) to form conjugates, the pellet was resuspended 10 times using a Pipetman set at 25 μl. Only 7 μl of this sample was needed for miscroscopic examination. The %conjugate formation was determined by the %T cells which were bound to fluorescent TC. Repeat counts of identical samples were within 2% of each other. A total of 200 to 400 T cells were counted per sample. The %conjugate formation with the same T cells and nonspecific BW5147.3 (*H-2*^{k}) TC varied from 0.5–3.5% from experiment to experiment. Similar results were obtained with three different preparations each of CP, H-2^k, and H-2K^b in a total of six experiments.

RESULTS AND DISCUSSION

The H-2K^b antigen, purified on the B8-24-3 mAb affinity column, was assessed to be greatly enriched by one-dimensional polyacrylamide (10%) gel electrophoresis (Fig. 1, lane 2). The two main protein species stained by silver stain were the heavy (M_r = 47,000–48,800) and light (M_r = 13,400) chains of H-2K^b. There is a slight amount of contamination with a protein, most likely actin, at M_r = 45,000 and a protein at M_r = 14,500. Two-dimensional gel analysis showed the presence of two major and two minor isoelectric species of the H-2 heavy chain (data not shown), consistent with conclusions of earlier studies showing charge differences due to sialylation (39).

Theoretically, 20 μg of H-2K^b could be isolated from cell surface of 10^9 EL4 cells, using 2.0×10^5 H-2K^b sites/cell (25). In fact, the yield of isolated antigen was 12–14 μg/10^9 cells.

The H-2^k antigens were purified as control H-2 proteins for the inhibition of conjugate formation experiments. One-dimensional gel analysis (Fig. 1, lane 1) of H-2^k indicated a pure preparation with heavy chain (M_r = 48,200–51,000) and light chain (M_r = 12,100).

The four major CP species isolated from BW5147.3 cells exhibited mobilities corresponding to M_r = 69,000, 67,700, 45,600, and 34,200 (see solid arrows in Fig. 1, lane 3). Variably, there were contaminants of the presumed H-2^k heavy- and light-chain proteins (see open arrows, lane 3, Fig. 1). A two-dimensional gel of CP isolated by essentially this same method from EL4 has already been published (25).

The lipid composition for the vesicles was chosen for two reasons: (i) a minimum

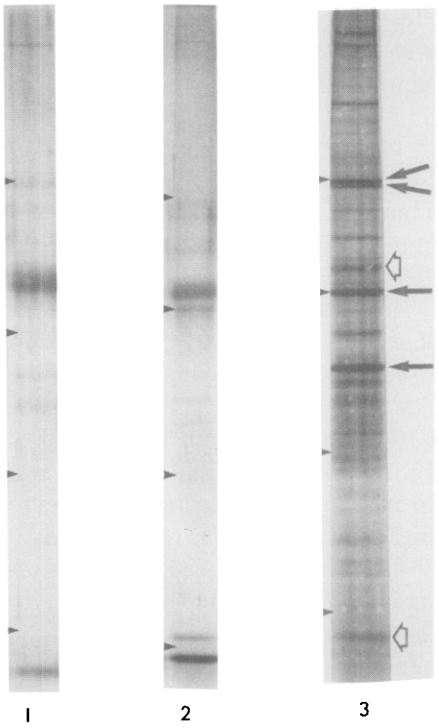

FIG. 1. One-dimensional sodium dodecylsulfate–polyacrylamide (10%) gel electrophoresis of mAb-purified H-2 preparations and cytoskeletal protein (CP) preparations: 1.2 μg purified H-2^k, purified on M1/42 affinity column (lane 1); 1.4 μg purified H-2K^b from EL4, purified on B8-24-3 affinity column (lane 2); 3.7 μg CP from BW5147.3 cells (lane 3). In lane 3, the solid arrows indicate the major CP species, and the open arrows indicate the heavy and light chains of the putative H-2^k contaminants. Arrowheads indicate positions of protein standards. The two larger protein standards were identical in all three lanes: bovine serum albumin (M_r = 68,000) and ovalbumin (M_r = 45,000). The two smaller protein standards were chymotrypsinogen A (M_r = 25,600) and ribonuclease A (M_r = 13,700) in lane 1 and trypsinogen (M_r = 24,000) and lysozyme (M_r = 14,300) in lanes 2 and 3. The two diffuse bands corresponding to M_r 60,000 and 66,000 were artifacts of the silver staining procedure and also appeared in lanes containing only the Laemmli (51) solution.

of 0.20 mol fraction cholesterol increases the lateral diffusion constant of membrane-embedded proteins (40), and (ii), ^{2}H-nuclear magnetic resonance studies have demonstrated that cholesterol tends to disorder the solid-phase lipid bilayer, thereby increasing the membrane fluidity (41).

The effect of the CP in the incorporation of H-2 into vesicles was examined by sucrose density-gradient centrifugation. Fractions containing vesicles, indicated by double-headed, horizontal arrows in Fig. 2, were identified by the simultaneous presence of both [^{14}C]cholesterol and ^{125}I-H-2K^b in the range of densities expected for vesicles containing protein and lipid (42). Protein which was not reconstituted with lipid would be expected in fractions numbers >19.

When CP were present, the vesicles were heavier and more heterogeneous in density (compare Figs. 2A and C). Also, most of the ^{125}I label was in the cholesterol-containing vesicles. However, CP-free vesicles contained a significant amount of ^{125}I-iodinated H-2K^b in a lighter fraction (No. 3), but no [^{14}C]cholesterol. It is possible that this light fraction contained either phospholipids and H-2K^b (but no cholesterol) or a trace of

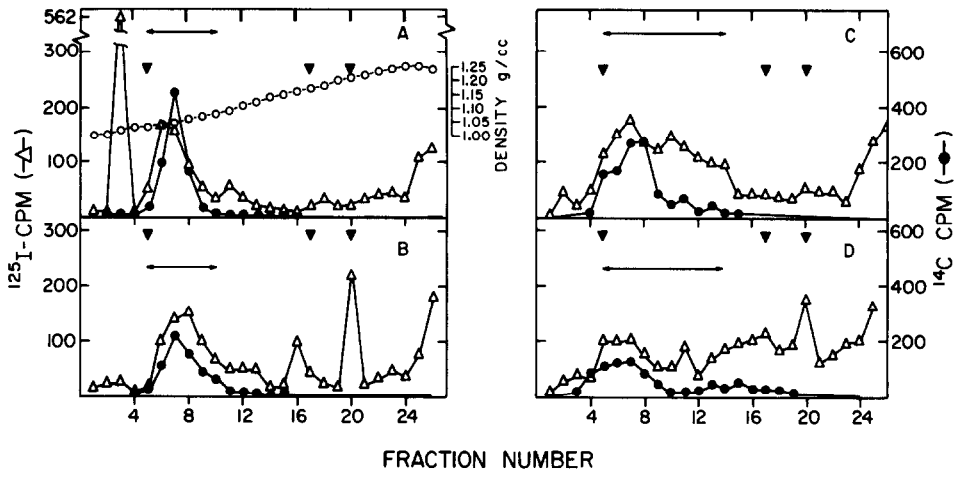

FIG. 2. Sucrose density-gradient (0–55%) profiles of vesicles containing tracer ^{125}I-iodinated H-2K^b (Δ) and tracer [^{14}C] cholesterol (●). Vesicles containing H-2K^b (2.5 μg) were prepared with the standard lipid composition either with or without CP (25 μg). They were pretreated with Pronase E or control buffer and subjected to sucrose density-gradient centrifugation. Fractions were collected from the top of the tubes and assayed for ^{125}I and ^{14}C to determine the amount of ^{125}I-H-2K^b and [^{14}C]cholesterol present, respectively. (A) Vesicles prepared from H-2K^b and lipid; (B) vesicles prepared from H-2K^b and lipid, pretreated with Pronase E; (C) vesicles prepared from H-2K^b, CP, and lipid; (D) vesicles prepared from H-2K^b, CP, and lipid, pretreated with Pronase E. A control sample was prepared by dialyzing out the detergent from a sample containing H-2K^b but no lipid and then applying it to the sucrose density gradient, as above. The inverted triangles (▼) indicate locations of peaks in this control tube at fractions 5, 17, and 20 containing 273, 105, and 784 cpm, respectively. All cpm values have been corrected for background. The density (O) of each fraction [see (A)] was calculated from the refractive index. The fractions which contained vesicles (see text) are indicated by a bar in each plot.

deoxycholate with H-2K^b. In a different study there was a hint of a separation of H-2K^k into two peaks on a sucrose density-gradient when reconstituted with cell lipids in the absence of CP (42), but neither cholesterol nor lipid was monitored. Our results imply that the CP aid in the incorporation of H-2K^b into cholesterol-containing vesicles.

To determine the degree of vectorial orientation of the H-2K^b antigen toward the outside of the vesicles (and, therefore, available for interaction with CTL), the vesicle preparations were pretreated with Pronase E, a general protease, and then centrifuged in the sucrose density gradient (Fig. 2B and D for CP-free and CP-containing preparations, respectively). Note that after Pronase E digestion there was an increase in ^{125}I-H-2K^b in fraction 20, consistent with proteolytic cleavage with resultant change in density. In a comparison of Fig. 2A and B essentially none of the antigen in CP-free vesicles was cleaved. This was surprising, but perhaps the lipid composition which we utilized affected the rate at which vesicles formed during detergent dialysis, thereby, affecting the mode of incorporation of H-2K^b into the vesicles (43). However, approximately 40% of the antigen in vesicles containing CP was cleaved by Pronase E (compare Fig. 2C and D). Papain cleavage studies on vesicles prepared either with or without CP showed that H-2K^k is slightly less susceptible to cleavage when CP are present (42). Assuming that the CP-containing vesicles were no more leaky to the Pronase E than vesicles formed in their absence, the data presented here suggest that the CP augmented the susceptibility of the cholesterol-and H-2K^b-containing vesicles

to proteolytic digestion and, thereby, aided in the vectorial reconstitution of H-2K^b into these same vesicles.

The ^{125}I-H-2K^b in fraction 3 (Fig. 2A) of the CP-free vesicles was no longer present after Pronase E treatment (Fig. 2B), indicating that it was totally cleaved by the enzyme. This supports the idea that some H-2K^b may have been in deoxycholate micelles. This light fraction was not observed in the sample containing CP (Fig. 2C).

Similarly prepared lipid vesicles containing H-2K^b (or H-2^k) and CP were used in the inhibition of conjugate formation experiments. The assay was adapted from that published previously (36) to increase the likelihood of successful inhibition by appropriately constructed vesicles. First, Ca^{2+} was omitted from the incubation buffer and EGTA was added to reduce the possibility of T-cell killing and recycling (44). Second, T cells were incubated with the vesicles for 1 hr at 37°C (45) before combining them with the TC. Third, a 2/1 T-cell/TC ratio was chosen in which the %conjugate formation was less than maximal to increase the sensitivity for the vesicles to inhibit conjugate formation.

The functional activity of reconstituted H-2K^b was judged by the amount of H-2K^b required (6 ng or 100 fmol) to yield 50% of the maximum observed inhibition of conjugate formation (Fig. 3). This amount of H-2K^b is 25- to 75-fold less than the amount necessary (150–450 ng) to stimulate a secondary cell mediated lysis response *in vitro* (13). Reproducible experiments were attained as long as freshly prepared vesicles were utilized.

Inhibition of conjugate formation was observed by the control vesicles reconstituted with H-2^k plus CP, but only at approximately five to sevenfold higher concentrations of H-2^k than observed with H-2K^b (Fig. 3). However, there was a clear separation of the responses as long as there was less than 15 ng H-2. This type of cross-reactivity was also observed in inhibition of conjugate formation containing fluorescently labeled EL4 TC and BALB/c anti-EL4 CTL by unlabeled P815 (*H-2^d*) or YAC (*H-2^a*) TC (38).

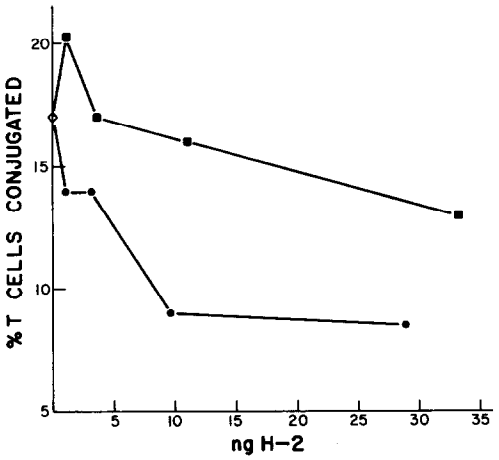

FIG. 3. Inhibition of T-cell–target cell conjugate formation by lipid vesicles reconstituted either with purified H-2K^b plus cytoskeletal proteins (●) or with H-2^k plus cytoskeletal proteins (■). Conjugate formation was measured by the %T cells conjugated to fluorescent TC. The %conjugate formation with the same T cells and nonspecific BW5147.3 (*H-2^k*) TC in this experiment was 2%. The final calculated ratio of CP to H-2 was 4.4/1 for H-2^k and 4.6/1 for H-2K^b. The amount of H-2 antigen indicated (as measured by recovered ^{125}I) is the amount added to the 7-μl volume that was analyzed under the epifluorescence microscope.

Nonspecific conjugate formation was assessed using third party *H-2*^{k} TC (BW5147.3) with CTL that had been primed to recognize *H-2*^{b} TC. Vesicles containing CP and H-2K^b only inhibit specific conjugate formation (Fig. 3) and had little effect on nonspecific conjugate formation (i.e., they reduced conjugate formation with BW5147.3 TC from 3.5 to 3.0%, using 22 ng H-2K^b per 7-μl sample).

The degree of cross-reactivity of the EL4 and BW5147.3 target cells with the CTL was not as strong as that observed when adding H-2 in model membranes. This may be because the H-2K antigenic density on the BW5147.3 cell [4.4×10^4 H-2K^k/cell; Ref. (46)] is less than that on the EL4 cell [2.0×10^5 H-2K^b/cell; Ref. (25)]. In contrast, the amount of H-2 antigens was quantitated precisely when adding them in vesicles.

Inhibition of conjugate formation by vesicles did not approach the limiting value (i.e., that observed with nonspecific TC), perhaps because there were T cells of such high affinity that vesicles containing reconstituted antigen could not compete with TC. Alternately, some of the allogeneically primed CTL might have been directed toward H-2D^b and, therefore, could not be inhibited by membrane-reconstituted H-2K^b. Other evidence suggests that these allogeneically primed T cells are primarily directed toward H-2K^b (32). Use of cloned CTL might help distinguish between these possible explanations.

The question of the specificity of the observed inhibition is of great importance. Vesicles containing H-2K^b inhibited conjugate formation between *H-2*^{b} TC and anti-H-2^b CTL while vesicles containing H-2^k did not (Fig. 3). This could be due to specific inhibition or could result from greater nonspecific inhibition by the H-2K^b vesicle preparation. These alternatives are usually distinguished by performing double-reciprocal experiments in which the same two-vesicle preparations would also be tested with *H-2*^{k} TC and anti-H-2^k CTL. However, nonspecific inhibition would most probably be caused by simple mechanical interference preventing effective CTL–TC interaction. Such interference would also be expected to have some influence on the nonspecific binding of cells since nonspecific binding forces are also subject to mechanical interference. Neither of the vesicle preparations had any effect on the nonspecific levels of binding. This suggests that the observed inhibition by H-2K^b vesicles was, in fact, specific at the concentrations used, and also supports the likelihood that the inhibition by H-2^k vesicles at higher concentrations was a cross-reaction of the type noted elsewhere with intact cells (38). In addition, the effects of nonspecific mechanical interference would be expected not to plateau at a level higher than nonspecific binding, as did the inhibition observed, but to reduce conjugate formation progressively to that of nonspecific binding (47).

An important consideration is that inhibition of conjugate formation is a relatively short-term assay, requiring only a 1-hr incubation at 37°C of vesicles with the T cells rather than a 5-day incubation, as needed in the secondary stimulation assay. The composition of the vesicles is less likely to be altered in such a short-term assay.

In comparison with our earlier work 180 ng purified plasma membranes [based on the revised yield of plasma membranes in Ref. 39] in a 7-μl sample was required to inhibit conjugate formation (15). Now using 6 ng H-2K^b plus 28 ng CP, matrix proteins, similar inhibition was observed. This is fivefold less total protein with the reconstituted vesicles.

One hundredfold more vesicular H-2K^b was required than is present on the TC to observe 50% inhibition. This decrease in efficiency can be attributed to any one of the following factors: (i) only partial vectorial orientation of the H-2K^b in the vesicles

(Figs. 2C and D), (ii) the absolute purity of the H-2K^b (Fig. 1), (iii) the difference in efficiency of vesicles and whole cells binding specifically to the CTL (48), and (iv) a suboptimal lipid composition, either for incorporation of H-2K^b into vesicles or for display of the antigens to the CTL.

The T cell/target cell ratio used in this work is sufficient to result in lysis. At a 2.5/1 T cell/target cell ratio using EL4 target cells and T cells raised identically as in this study, 14% specific ^{51}Cr release is observed in a 90-min assay (36). When the BW5147.3 cell is the target cell, no ^{51}Cr release is observed (36). Additionally, using these same T cells at a 10/1 ratio, we have observed 50% specific fragmentation of EL4 target cell DNA (49) in a 30-min assay (data not shown).

The ability of these reconstituted vesicles to inhibit the lytic event, as monitored by ^{51}Cr release, has not been tested, primarily because plasma membrane vesicles were not effective inhibitors (Gilmer, unpublished results). Studies currently under way suggest, however, that appropriate purified plasma membranes inhibited specific DNA fragmentation while third-party membranes did not (Figard and Gilmer, unpublished results).

However, since the amount of purified H-2 required is relatively small, this inhibition of conjugate assay can be used to test how such parameters such as the lipid composition, membrane fluidity, surface charge, subcellular source of the antigens, and density of the antigens affect conjugate formation. In this way the parameters that are critical for H-2 recognition by CTL can be evaluated and optimized. Recently, flow cytometry has been utilized to monitor conjugate formation, and inhibition of conjugate formation by reconstituted antigen will be monitored using this technique (50).

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