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Immunology

Allogeneic stimulation of cytotoxic T cells by supported planar membranes

(class I major histocompatibility antigen/membrane reconstitution/antigen processing/LFA-1 cell surface protein/lateral diffusion)

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ABSTRACT **Phospholipid vesicles containing the transmembrane protein H-2K^k spontaneously fuse to form planar membranes when incubated on treated glass surfaces. Pattern photobleaching of fluorescent lipid probes indicates that these planar membranes are continuous and that the lipids are as mobile as they are in conventional fluid bilayers or monolayers. H-2K^k molecules in these planar membranes are immobile. These membranes stimulate cytotoxic T lymphocytes when cultured with immune spleen cells. The response to H-2K^k in planar membranes is greatly enhanced by the addition of supernatant from concanavalin A-stimulated spleen cells, indicating that relatively little antigen processing or presentation by accessory cells occurs. Cytotoxic T cells induced by purified alloantigen are found to be as susceptible to antibody blockade as are effectors from conventional mixed lymphocyte culture, where the antibody is directed against a T-cell surface antigen reputed to strengthen target cell adhesion through an interaction independent of major histocompatibility antigens.**

Gene transfer techniques allowing the expression of isolated genes in a new and defined cellular environment have made it possible to associate defined cellular functions with specific molecular entities. Recently, for example, the ability to present antigen to antigen-specific, major histocompatibility complex (MHC)-restricted T-cell hybridomas was conferred on mouse L cells by the transfer and expression of cloned IA^k genes (1). Thus, the minimal requirement for presentation of antigen would appear to be expression of Ia molecules on accessory cells.

One approach to an understanding of the molecular basis of MHC-restricted immune recognition and triggering is to study MHC proteins and foreign antigens in the still further defined environment of a synthetic lipid bilayer. Recent work in this laboratory has been directed toward developing techniques capable of providing information on the behavior of molecules in cell membranes and in model membranes during specific adhesion and triggering. Total internal reflection techniques enable one to observe exclusively fluorescently tagged molecules located within the area of contact between a cell and a planar model membrane (2). With a view toward applying this method to H-2-restricted recognition by T cells, we have reconstituted H-2K^k in phospholipid monolayers spread at an air/water interface and shown that they specifically bind cloned cytotoxic T cells (3).

In this report we describe a greatly simplified method for the construction of continuous planar membranes. Although at present these membranes are less well defined physically than membranes described previously (3–5), they can easily be made to contain protein and can be made sterile. We show that planar membranes containing H-2K^k evoke a specific cytotoxic response from spleen cells from previously immunized mice. Although stimulated with purified antigen, effector cells from these cultures are inhibited by antibodies to T-cell proteins that mediate antigen-independent adhesion. An interesting feature of this system is that, unlike antigen reconstituted into vesicles or liposomes, H-2K^k in planar membranes is apparently not processed and presented by accessory cells.

MATERIALS AND METHODS

Mice and Culture Media. Responder mice for cytotoxic T lymphocyte (CTL) stimulation experiments were B10.AQR (H-2K^q, I A,E^k, H-2D^d) obtained from a colony maintained at Stanford University by Hugh McDevitt. A/J stimulator mice (H-2K-IE^k, H-2D^d) were obtained from the Institute for Medical Research (San Jose, CA). B10.A stimulator mice were from The Jackson Laboratory. Spleen cells were cultured in Costar 12-well plates in RPMI 1640 medium supplemented with nonessential amino acids at 100 μM each, 50 μM 2-mercaptoethanol, penicillin at 100 units/ml, streptomycin at 100 μg/ml, 1 mM glutamine, 1 mM sodium pyruvate, and 10% fetal calf serum. Tumor cells were maintained in medium lacking 2-mercaptoethanol and nonessential amino acids. Supernatant from Con A-stimulated rat spleen cells (Con A supernatant) was made as described (6) and was passed over a small column (approximately 0.5 ml of bed volume per 10 ml of supernatant) of Sephadex G-75 (Pharmacia) to remove residual Con A and was diluted to 80% with medium.

H-2K^k and Antibodies. H-2K^k was purified from RDM4 cells grown as ascites in AKR/J mice using a method previously described (7). Monoclonal 11-4.1 antibody (anti-H-2K^k) (8) was purified from culture supernatants by affinity chromatography on staphylococcal protein A. Fluoresceination of H-2K^k was carried out by dialyzing the purified protein against a solution of 50 mM sodium carbonate/bicarbonate buffer, pH 9.2, 140 mM NaCl, and 0.5% sodium deoxycholate, then stirring the dialyzed protein with a 10:1 mole excess of fluorescein isothiocyanate (Molecular Probes, Junction City, OR) in the dark at room temperature for 4 hr. The reaction mixture was then dialyzed in the cold against buffer containing 140 mM NaCl, 0.5% deoxycholate, and 10 mM Tris·HCl, pH 8, until the dialysate was colorless. Residual fluorescein was removed by affinity chromatography on lentil lectin. The lentil lectin column was washed until fluorescein was not detected in the wash buffer by using a hand-held long-wavelength UV lamp. H-2K^k was then eluted with buffer containing 5% methyl α-D-mannoside. Fluoresceination of 11-4.1 was carried out in 100 mM carbonate/bicarbonate buffer, pH 9.2, with a 10:1 mole excess of fluorescein isothiocyanate. After reaction, free fluorescein was removed by exhaustive dialysis against phosphate-buffered saline, pH 7.2, (P_i/NaCl). The resulting fluorescein-to-protein mole ratio for antibody used in these ex-

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Abbreviations: CTL, cytotoxic T lymphocyte; MHC, major histocompatibility complex; NBD-Myr$_2$-PtdEtn, *N*-4-nitrobenzo-2-oxa-1,3-diazole-dimyristoyl phosphatidylethanolamine.

periments was 7:1. Culture supernatant was used as the source of the monoclonal antibody I21/7.7 (9), which recognizes an antigen having the properties of LFA-1 with respect to molecular weight, quaternary structure, and CTL function (10).

Lipid Vesicles. Stock solutions of egg phosphatidylcholine (Sigma) and cholesterol (recrystallized three times from ethanol) were combined in a glass test tube at a 7:2 mole ratio. For photobleaching *N*-4-nitrobenzo-2-oxa-1,3-diazole-dimyristoyl phosphatidylethanolamine (NBD-Myr_2-PtdEtd) (Avanti Polar Lipids) was added to give 1 mol %. After evaporation of solvent under a stream of nitrogen the tube was kept under reduced pressure for 15–30 min. The lipids were then dissolved in a solution containing 140 mM NaCl, 10 mM Tris·HCl at pH 8, and 0.5% deoxycholate. The solution was bath sonicated to clarity and then diluted into a solution containing H-$2K^k$ in the same buffer or into buffer solution without protein. The H-$2K^k$ concentration was 10–20 μg/ml. Reconstitution was carried out at a lipid-to-protein ratio of 10:1 (wt/wt) unless otherwise stated. Vesicles were formed by dialysis against four 1-liter changes of P_i/NaCl for 84 hr at 4°C. If the vesicles were to be used for stimulation of CTL the lipid/detergent solution was passed through a sterile 0.2-μm-pore filter (Gelman Acrodisc), loaded into sterile dialysis tubing, and dialyzed under sterile conditions.

Photobleaching and Fluorescence Microscopy. Membranes were observed by epi-illumination with the 488-nm line of an argon ion laser. Lateral diffusion coefficients were calculated from fluorescence recovery after photobleaching, using a Ronchi ruling (11, 12). Patterns used had a period of 7.9 μm and 12.7 μm. Photobleaching was carried out with a laser power of 175 mW.

Formation of Planar Membranes. Prior to use coverslips (18 mm round, no. 1 thickness) were boiled in Linbro 7× detergent (Flow Laboratories) at a 1:4 (vol/vol) dilution, rinsed overnight under running deionized water, then dried in an oven at 120–150°C for 1 hr. Finally, coverslips were cleaned in an argon plasma cleaner (Harrick) for 15 min. For alkylation, coverslips were dipped in a bath containing hexadecane/carbon tetrachloride/chloroform (90:6:4, vol/vol) and 100 μl of octadecyltrichlorosilane per 100 ml until they readily shed excess solution. After alkylation, coverslips were rinsed several times in chloroform, then air dried overnight.

For microscopy, planar membranes were made by placing a coverslip, alkylated or unalkylated, on top of 80 μl of vesicle suspension in a clean glass Petri dish for 10 min at room temperature. After incubation the Petri dish was filled with P_i/NaCl and the coverslip, membrane side down, was attached to a clean glass microscope slide by two strips of double-stick tape. The membrane was then washed by allowing at least one Pasteur pipette-full of P_i/NaCl to flow between the membrane and the microscope slide. Membranes were never exposed to air. Microscopy was carried out on samples immersed in P_i/NaCl, using a ×40 water-immersion objective.

Before incubation with antibodies the membranes were washed with P_i/NaCl containing 0.2% serum. Thirty microliters of fluorescent antibody (previously centrifuged at 100,000 × *g* for 20 min to remove aggregates) at about 20 μg/ml in the same buffer solution was added to the space between the membrane and the microscope slide. After a 10-min incubation the membrane was washed with P_i/NaCl/0.2% serum.

Coverslips and microscope slides used for production of planar membranes for cell culture were sterilized by soaking in absolute ethanol. Membranes used for CTL stimulation were made by holding coverslips on 80-μl droplets of vesicle suspension on microscope slides for 10 min at room temperature. To deliver the coverslips to the bottoms of culture wells membrane-side-up, culture wells were first filled to brimming with warm (≈37°C) medium (7.4 ml). Then, resting one end of the slide on the rim of the well, the slide was quickly pivoted. At no time was the supported membrane exposed to air. Before adding cells and medium containing serum, 6.7 ml was carefully removed from each well.

***In Vitro* Stimulation of CTL.** Responder mice were immunized 1–3 months before use by intraperitoneal injection of 10^7 spleen cells from A/J or B10.A mice. Spleen cells from responder mice were suspended in medium containing 20% fetal calf serum. Stimulator spleen cells, either A/J or B10.A, were irradiated with 3000 rads (1 rad = 0.01 gray) and suspended in medium containing 10% serum. Cultures were set up by introducing 0.2 ml of medium or medium with stimulator cells and 0.6 ml of responder cell suspension to wells containing planar membranes made with or without H-$2K^k$. Positive control wells contained stimulator cells, responder cells, and planar membranes made from H-2-free vesicles. Negative control wells contained H-2-free planar membranes and responder cells. Eighteen to 24 hours after culture had been initiated, wells were given an additional 0.5 ml of medium with 10% serum or 0.5 ml of 80% Con A supernatant. Cytotoxicity was tested on day 5 against ^{51}Cr-labeled RDM4 (H-2^k) cells and P815 cells or bacterial lipopolysaccharide-induced B10.AQR blasts in a 4-hr assay with 10^4 target cells per well in U-bottom microtitration plates. The percent lysis is calculated from the expression % lysis = $100 \times (E - B)/(T - B)$, for duplicate samples, in which E is the cpm released into the supernatant in wells containing effectors, B is the background release in the absence of effectors, and T is the cpm released from target cells by 0.5% nonionic detergent.

Antibody Blocking. The effect of I21/7.7 cytotoxicity was determined at a constant effector-to-target ratio (40:1) for effectors stimulated with either B10.A or planar membranes made with H-$2K^k$, using antibody at various dilutions in a ^{51}Cr release assay. The percent inhibition is expressed as (1 − Exp/Ctrl) × 100, in which Exp is lysis in the presence of antibody and Ctrl is lysis without added antibody.

RESULTS

Formation of Planar Membranes on Glass Surfaces. When alkylated or unalkylated coverslips are examined by fluorescence microscopy after incubation with lipid vesicles containing 1 mol % NBD-Myr_2-PtdEtd, a fluorescent focal plane can be found at the level of the undersurface of the coverslip. No fluorescent layer can be found if nonfluorescent vesicles or P_i/NaCl is used, indicating that the observed fluorescence is not from the coverslip or microscope slide underneath. Uniform fluorescent membranes are formed when the vesicle suspension contains lipids at 2, 0.2, or 0.1 mM, but not when the vesicle suspension contains 0.01 mM lipids. The fluorescent membrane appears uniform with occasional small vesicles attached. Lateral diffusion coefficients of NBD-Myr_2-PtdEtd in planar membranes measured by fluorescence recovery after photobleaching on alkylated and nonalkylated surfaces are $2.10 \pm 0.26 \times 10^{-8}$ cm^2/sec and $3.71 \pm 0.15 \times 10^{-8}$ cm^2/sec, respectively. On both surfaces the fluorescence recovery is 80–98% of the theoretical maximum.

Incubation of vesicles containing H-$2K^k$ with alkylated or nonalkylated coverslips results in planar membranes that bind fluoresceinated anti-H-$2K^k$ antibody specifically. The fluorescence observed under epi-illumination is bright and uniform. No bound fluorescence is observed when 11-4.1 is added to planar membranes lacking H-$2K^k$. Irrelevant antibodies, such as fluoresceinated anti-nitroxide-dinitrophenyl hapten, do not bind to membranes containing H-$2K^k$. The diffusion coefficient of NBD-Myr_2-PtdEtd in membranes with H-$2K^k$ is $3.50 \pm 0.12 \times 10^{-8}$ cm^2/sec. Recovery values

range from 74% to 86% of the maximal possible recovery. Patterns bleached onto planar membranes containing fluoresceinated H-2K^k or fluoresceinated 11-4.1 bound to H-2K^k persist for several minutes, indicating that H-2K^k is virtually immobile. Even when planar membranes contain H-2K^k at a 100:1 lipid-to-protein ratio (wt/wt), no measurable diffusion of fluorescent 11-4.1 occurs.

Stimulation of Secondary CTL by Using Planar Membranes. To assess further the structural integrity of H-2K^k molecules, planar membranes made on alkylated and unalkylated surfaces were placed in culture with spleen cells from previously immunized B10.AQR mice. Stimulating cultures under various conditions, we found that responses to planar membranes were generally stronger at higher cell densities, were stronger when the coverslip was unalkylated, and were usually dependent on the presence of added growth factors. Killing of syngeneic B10.AQR blasts was never more than a few percent above spontaneous release backgrounds. Lysis of third-party P815 cells by antigen-stimulated cells was not greater than lysis by mock-stimulated cells. Fig. 1 shows the effect of coverslip alkylation on the magnitude of the response. The experiments shown were carried out with two different batches of coverslips at two different times with either 3×10^6 or 6×10^6 responding B10.AQR cells per well. In both experiments responder cells cultured on membranes made on unalkylated coverslips were more cytotoxic than cells cultured under identical conditions on membranes made on alkylated coverslips. The difference in response cannot be ascribed to a consistently inhibitory effect of alkylation because positive control cells cultured on alkylated coverslips do not show a consistently lower response than positive control cells cultured on unalkylated coverslips.

The experiment shown in Fig. 2 demonstrates the dependence of the response on exogenous factors. Added Con A supernatant does not significantly affect the response of B10.AQR cells against H-2K^k on B10.A cells. On the other hand, the response from cells cultured on planar membranes containing H-2K^k is increased substantially by the presence of exogenous factors. This effect requires the presence of antigen. Cells cultured in the absence of antigen are only weakly cytotoxic whether given factors or not. Occasionally, cells cultured at high density (1.2×10^7 per well) show strong responses to H-2K^k in planar membranes in the absence of added factors, but in nearly all experiments, at different cell densities, addition of Con A supernatant resulted in significantly greater cytotoxicity. The action of added factors is not to overcome any immunosuppressive effects of the planar membranes. Supernatants taken from cells cultured on H-2K^k planar membranes and not given Con A supernatant are not suppressive.

In a number of experiments (data not shown) we consistently found that the above planar membranes were far more efficient for CTL stimulation than were comparable amounts of lipid and protein in the form of vesicles produced by dialysis.

Antibody Blocking. The effect of I21/7.7 on cytotoxicity is shown in Fig. 3. Despite the generally lower lytic capability of cell populations stimulated with planar membranes compared to cells stimulated with antigen-bearing cells, there is only a slight difference in their susceptibility to blocking by I21/7.7. Neither population was appreciably inhibited by anti-Lyt 2.2 (data not shown). The curve for the B10.A stimulated population does not extrapolate to 100% inhibition, possibly reflecting the presence of killers with natural killer-like activity. The antibody dilution resulting in 50%

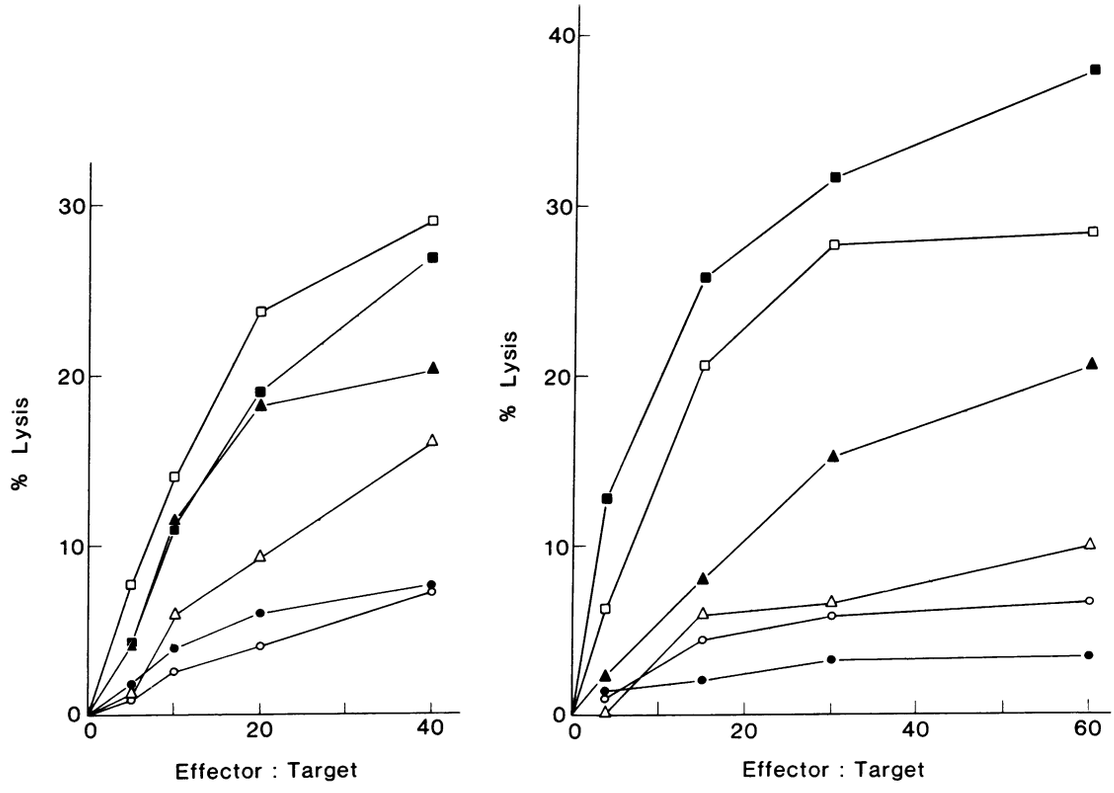

FIG. 1. Effect of glass pretreatment on CTL response. Cells were incubated on alkylated (open symbols) or unalkylated (closed symbols) coverslips supporting planar membranes containing H-2K^k at a 10:1 lipid-to-protein ratio (△, ▲) or made with lipids only. □ and ■, wells given 3000-rad γ-irradiated A/J; ○ and ●, wells with no stimulator cells. (*Left*) Wells contained 6×10^6 B10.AQR responders. Positive control wells contained, in addition, 6×10^5 A/J stimulators. (*Right*) Wells contained 3×10^6 B10.AQR responders and positive control wells also contained 6×10^5 A/J.

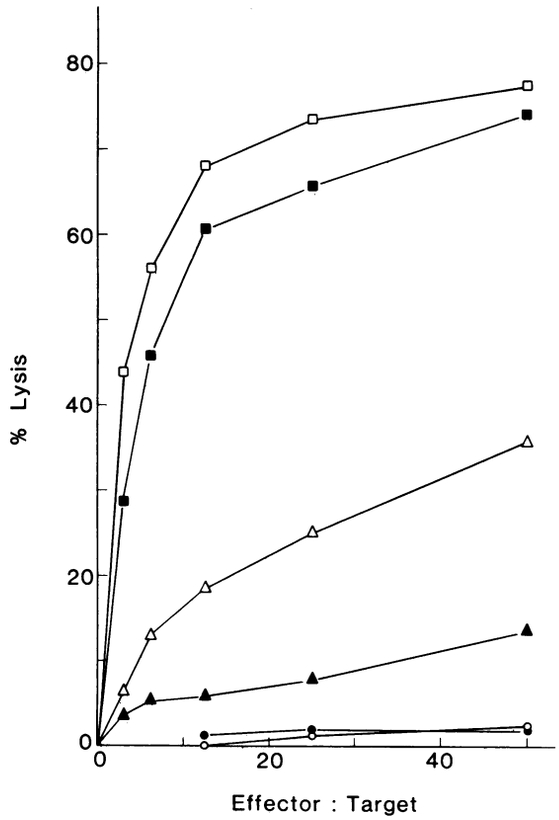

FIG. 2. Effect of added factors on CTL response. Open symbols, wells given Con A supernatant. □ and ■, wells containing 1.2×10^7 B10.AQR along with 1.2×10^6 B10.A (3000-rad irradiated) and an unalkylated coverslip supporting a planar membrane made without H-2K^k. ○ and ●, same as above but without B10.A. △ and ▲, wells containing 1.2×10^7 B10.AQR and a planar membrane made with H-2K^k at a 3:1 lipid-to-protein ratio.

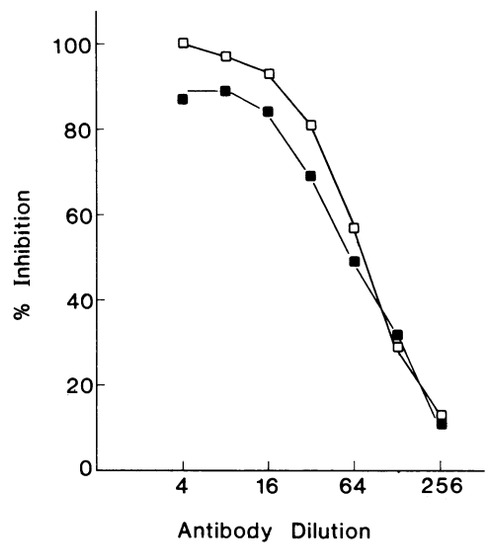

FIG. 3. Inhibition of cytotoxicity by I21/7.7 antibody of lysis of RDM4 targets by B10.AQR effectors cultured at 1.2×10^7 on planar membranes containing H-2K^k (□) or on planar membranes made without H-2K^k along with 1.2×10^6 B10.A γ-irradiated with 3000 rads (■). Cultures were given Con A supernatant approximately 18 hr after initiation.

inhibition was found to be 1:80 for the planar membrane-stimulated population and 1:60 for the B10.A-stimulated population.

DISCUSSION

The photobleaching measurements of diffusion of NBD-Myr$_2$-PtdEtd show that a continuous membrane is formed when lipid vesicles are incubated briefly with alkylated or unalkylated coverslips. The fluorescent focal plane observed cannot arise from dense packed vesicles adhering to the surface because diffusion occurs over distances much greater than the dimensions of ≈1000-Å-diameter individual vesicles. The measured diffusion coefficient is in the range of values expected for diffusion of phospholipid molecules in fluid bilayers (13) or monolayers made at an air/water interface (5). When the sample is bleached without a grid in place, no significant recovery occurs, indicating that diffusion coefficients measured are not an artifact of exchange equilibrium between the surface and the solution. H-2K^k in these membranes does not show measurable diffusion, unlike its behavior in liposomes, where a diffusion coefficient of 1.13×10^{-8} cm^2/sec has been measured (14). The uniform appearance of the fluorescence from fluoresceinated H-2K^k or fluoresceinated 11-4.1 indicates that the proteins are not highly aggregated. As demonstrated by the specific binding of antibodies, at least some of the H-2K^k molecules are oriented right side out, and, if more than one layer is present, are in the outer layer exposed to the aqueous medium.

A more stringent test of the integrity of H-2K^k molecules in planar membranes is provided by the CTL stimulation assays. These experiments show clearly that the H-2K^k is recognized by precursor cytotoxic T cells. The amount of H-2 present in these membranes is controlled by the lipid-to-protein mole ratio in the initial reconstitution. Membranes used in the experiments shown in the figures contain lipid and protein at nominal ratios of 10:1 or 3:1 by weight (not including cholesterol). Assuming an average molecular weight of 1000 for egg phosphatidylcholine, the resulting lipid-to-protein mole ratio is 600:1 (Fig. 1) or about 200:1 (Fig. 2). Using these numbers, it is possible to obtain a rough estimate of the amount of H-2 in these planar membranes. Assuming on average that one phospholipid molecule occupies an area of 60 Å^2 (15), an 18-mm-diameter coverslip will be covered with about 4×10^{14} lipid molecules. The number of H-2K^k molecules on a coverslip coated from vesicles reconstituted from a 10:1 mixture is about 7×10^{11}, or about 3000 molecules per μm^2. For a molecular weight of 60,000 this number of molecules is equivalent to about 0.07 μg, or roughly 4×10^5 cell equivalents, according to Herrmann and Mescher (7). This calculation assumes that the coverslip is coated with a molecular monolayer. For a bilayer, the amount of H-2 present must be twice this. On the basis of this calculation the amount of H-2K^k available in the experiments shown in Figs. 1 and 2 is roughly equivalent to that present on the B10.A or A/J stimulator cells in the positive control wells. On the other hand, we have no independent experimental evidence regarding the amount of lipid on the coverslips. If all of the lipid contained in an 80-μl droplet were deposited onto the glass, the planar membrane would be about 10 bilayers thick.

Several cell surface proteins such as LFA-1 and Lyt-2,3 have been shown to serve in accessory roles in T-cell-mediated cytotoxicity. The blockade mechanism of antibodies to these proteins is apparently in the binding stage rather than in delivery of the lethal hit (10). LFA-1 and the possibly identical 170/100 complex recognized by I21/7.7 are broadly distributed among leukocytes and may have some general function in cell–cell adhesion (9, 16). When responder cells are cultured with purified reconstituted antigen, stabilizing interactions through protein ligands other than antigen are unavailable to precursor cytotoxic cells. For this reason, and

because of our interest in using model membranes to study T-cell–target membrane interactions, we asked whether differentiated CTL derived by immunization with purified antigen would differ from their more conventionally derived counterparts with respect to their dependence on antigen-independent interactions. The data shown in Fig. 3 indicate substantial inhibition by I21/7.7 of the cytotoxicity of both CTL populations. This result suggests that immunization with purified reconstituted antigen does not select an unusual subset of CTL. Although these data do not speak directly to the question of relative receptor–MHC antigen affinities they show that these two populations of CTL show no significant difference in their dependence on at least one adhesion-strengthening interaction independent of MHC antigens.

One significant difference between these experiments and other work using H-2K^k reconstituted into vesicles (17–19) is the dependence seen here on added growth factors. Apparently the H-2K^k is recognized by pre-CTL because a significant response occurs only when membranes contain antigen; however, the requirement for exogenous factors indicates that H-2K^k in planar membranes does not stimulate T-cell help. The most likely explanation for this is that antigen in these membranes is not processed by antigen-presenting cells. It has been shown that antigen presentation by accessory cells is a necessary step for the stimulation of cytotoxic responses by H-2K^k in liposomes or vesicles (18). This requirement is obviated by the addition of factors from mitogen-stimulated lymphocytes. Work from several laboratories has shown that helper T cells recognize antigen in denatured and degraded form along with self-Ia molecules on antigen-presenting cells (20, 21). Evidently little degradation of antigen in planar membranes by accessory cells occurs. This effect is not likely to be due to inhibition of antigen processing because responses against B10.A or A/J cells cultured on lipid membranes lacking H-2K^k are not similarly dependent on added factors.

A plausible explanation for the lack of T-cell help is that accessory cells are able to degrade large, native proteins only after they are internalized. Lysosomotropic agents such as chloroquine and ammonium chloride inhibit presentation of protein antigens (22, 23). In the course of a mixed lymphocyte reaction, degradation of alloantigen by accessory cells may occur after phagocytosis of small, subcellular vesicles or membrane fragments released by the breakdown of dead cells or shed normally from live cells (24, 25). If only small fragments were readily internalized and planar membranes were sufficiently stable in culture very little antigen would be available to accessory cells for degradation and subsequent presentation to helper T cells.

The low diffusion coefficient of H-2K^k in our planar membranes may be due to the strong interaction of this transmembrane protein with the underlying substrates. Unfortunately this has prevented our determination of whether laterally mobile H-2K^k molecules would be more or less efficient in stimulating the CTL response. In future work it will be desirable to find conditions under which the diffusion coefficients of integral membrane proteins can be controlled experimentally, so as to answer this question, and to facilitate other biophysical studies of interactions between membranes. The use of lipid-tethered peptides and antigens is one approach to this problem (26).

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