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CYTOTOXIC T CELL ACTIVATION BY CLASS I PROTEIN ON CELL-SIZE ARTIFICIAL MEMBRANES: ANTIGEN DENSITY AND LYT-2/3 FUNCTION[1]

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The role of antigen surface density, and its relationship to the function of the Lyt-2/3 complex, in recognition and triggering of allospecific cytotoxic T lymphocyte (CTL) precursors has been studied by using a novel type of Class I protein-bearing artificial membrane. The cell-size membranes, termed pseudocytes (artificial cells), can be handled like cells but have a well-defined and easily quantitated surface composition. Class I antigen on these membranes stimulated generation of secondary in vitro allogeneic CTL responses as effectively as allogeneic spleen cells, provided that lymphokines were added to the cultures. Antigen density on the pseudocyte surfaces could be varied over a wide range and quantitated by papain cleavage and fluorescence-activated cell sorter analysis. Recognition and triggering of precursor CTL was found to be dramatically dependent on the surface density of antigen and displayed a marked threshold density requirement, below which little or no response occurred. Examination of the effects of anti-Lyt-2 antibody on responses to pseudocytes provided direct evidence for a reciprocal relationship between antigen density and susceptibility to antibody blockade. The results strongly suggest that antigen density is likely to have important biological consequences in control of immune responses. They also show that if Lyt-2/3 functions by interaction with a ligand, then that ligand is the Class I protein.

Cytotoxic T lymphocytes (CTL) recognize Class I major histocompatibility complex (MHC) proteins on the surfaces of other cells (1, 2). Results from in vitro studies (3–8) suggest that CTL recognition of allogeneic Class I molecules is affected not only by the presence of polymorphic Class I determinants, but by variations in levels of cell surface expression of the Class I proteins as well. Indeed, decreased levels of Class I antigen expression were reported to provide tumor cells with a selective advantage in vivo (9, 10). Studies to assess the influence of Class I antigen surface density on recognition, however, have met with limited success because of difficulties inherent in attempts to vary, and to quantitate, the level of Class I antigen expression on metabolically active cells.

In previous studies researchers have compared CTL-mediated lysis of cells expressing differing amounts of H-2 antigen as a result of modification by interleukin-induced activation (8), by in vivo vs in vitro passage (3–5), or by enzymatic cleavage of surface proteins (6, 7). Interpretation of these studies is complicated for several reasons. First, monoclonal target cells that have been treated differently vary in surface membrane composition parameters other than just H-2 antigen surface density. Such differences can significantly effect CTL recognition and susceptibility of a cell to CTL-mediated lysis even when levels of H-2 antigen expression remain unchanged (4, 11–14). Second, surface membrane composition can change rapidly on metabolically active cells. Thus, papain-treated target cells recovered susceptibility to lysis within minutes after being placed in culture with effector CTL (7). So rapid a change prevents accurate definition of the membrane composition at the time that functional recognition is occurring. Finally, because the target cell ligands for Lyt-2/3, LFA-1, and any other accessory CTL molecules that might function in lysis remain undefined, their levels and influence cannot be assessed. It is not surprising, therefore, that the dependence of T cell recognition on levels of H-2 Class I expression remains poorly understood.

The Lyt-2/3 accessory molecule is particularly interesting with respect to consideration of the effects of antigen density on T cell recognition. Generation of effector CTL (15) and CTL-mediated target cell lysis (16, 17) can be inhibited by monoclonal antibodies (mAb)[3] specific for the Lyt-2/3 complex on murine CTL. The function of the Lyt-2/3 molecule and the mechanism by which antibodies against it block CTL are not known. However, CTL have been shown to be heterogeneous in their susceptibility to inhibition by anti-Lyt-2 mAb (18), and there is considerable evidence to indicate a reciprocal relationship between the ability of CTL to respond to antigen and their susceptibility to blockade (19). Thus, reducing the amount of antigen expressed on target cells by proteolytic cleavage increased susceptibility of CTL-mediated lysis to mAb blockade (7). In other work (8), lysis by some CTL clones was shown to require IFN-γ treatment of target cells to induce high levels of Class I protein expression, and these clones were more susceptible to anti-Lyt-2 mAb blocking than clones able to lyse

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[3] Abbreviations used in this paper: Con A supernatant, culture fluid from concanavalin A-stimulated rat lymphocytes; DMPC, dimyristoylphosphatidylcholine; DOC, deoxycholate; mAb, monoclonal antibody; STEM, scanning transmission electron microscopy; TBS, Tris-buffered saline; NP, nitrophenyl.

untreated target cells. It has been suggested (19, 20) that the Lyt-2/3 protein functions to stabilize CTL-target cell conjugates, and that this may occur via interaction with conserved determinants of Class I molecules on the antigen-bearing cell (21). An alternative, but not mutually exclusive, possibility is that antibody binding to Lyt-2/3 results in delivery of a negative signal to the T cell (22).

In this paper we describe experiments performed with a novel type of Class I alloantigen-bearing artificial membrane to examine the role of antigen surface density in triggering of precursor CTL responses and to study the function of Lyt-2/3 molecules. Recently, we reported preparation of the cell-size, supported artificial membranes, and we demonstrated that Class I antigen on these membranes stimulates in vitro generation of specific secondary allogeneic CTL responses as effectively as allogeneic spleen cells (23). The membranes, termed pseudocytes (artificial cells), can be handled like cells, but have the advantage of being composed of well-defined, readily varied, and easily quantitated components. In this study, the density of antigen on the pseudocyte surface was varied over a wide range and was quantitated by fluorescence-activated cell sorter (FACS) analysis. Alloantigen recognition and triggering of the secondary CTL response was found to be dramatically dependent on the density of antigen on the membrane surface. Examination of the effects of anti-Lyt-2 mAb provided direct evidence for a reciprocal relationship between antigen density and susceptibility to blocking. The results argue strongly that if Lyt-2/3 functions by interaction with a ligand, then the ligand is the Class I protein.

MATERIALS AND METHODS

Mice and tumor cells. (BALB/c × DBA/2)F_1 (CD2F_1) (H-2^d), C57BL/6 (H-2^b), AKR/J (H-2^k), and A/J (H-2^a) mice were purchased from The Jackson Laboratory, Bar Harbor, ME. RDM-4 (H-2^k), an AKR lymphoma, P815 (H-2^d), a mastocytoma of DBA/2 origin, and EL-4 (H-2^b), a C57BL/6 thymoma, were maintained in tissue culture and by passage as ascites.

Monoclonal antibodies and antisera. The mAb used in this study were directed against H-2K^k (11-4.1) (24), Thy-1.2 (HO-13-4) (25), and Lyt-2/3 (53-6.7) (26). Hybridomas were obtained from The Salk Cell Distribution Center and are now available from The American Type Culture Collection. An anti-nitrophenyl (anti-NP) idiotype mAb (R3-I), and a fluorescein isothiocyanate (FITC)-conjugated goat anti-murine γ_{2a} antisera were donated by Dr. Carol Cowing and Dr. Donald Mosier, respectively, of the Medical Biology Institute, La Jolla, CA. All antibodies were partially purified by ammonium sulfate fractionation except for the anti-Lyt-2/3 mAb, which was a culture supernatant having 40 μg mAb/ml.

Purification of Class I antigens. H-2K^k was purified from RDM-4 ascites cell detergent lysates by affinity chromatography on an 11-4.1 mAb column as described (27) with a yield of about 0.9 mg H-2K^k/10^{10} cells. H-2K^d/D^d and H-2K^b/D^b antigens were purified by affinity chromatography from P815 or EL-4 ascites cell detergent lysates, respectively, on an M1/42 mAb column as described (28), with a yield of about 0.25 mg H-2 antigen/10^{10} cells. All H-2 preparations were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 5 to 15% acrylamide slab gels used with the buffer system of Laemmli (29) with a 3% stacking gel. Protein was visualized by staining with Coomasie Blue. ^{125}I-H-2K^k was prepared by reacting 10 μg purified antigen with 0.5 mCi Na^{125}I (NEN, Boston, MA) and two Iodobeads (Pierce Chemical Corp., Rockford, IL) in 0.15 ml 0.5% deoxycholate for 30 min at 4°C, and was isolated by Sephadex G-25 chromatography and dialysis, yielding a product with a specific activity of about 300 cpm/ng protein.

Preparation and handling of pseudocytes. Spherisorb 5-μm ODS1 beads were purchased from Phase Sep, Norwalk, CT. Dry beads were suspended by vortex mixing in 0.5% deoxycholate in 10 mM Tris buffer, 0.14 M NaCl, pH 8.0 (DOC/TBS), were washed twice in this buffer by centrifugation (2 min, 1500 rpm), and were counted by using a hemacytometer. Lipids either were derived by chloroform/methanol extraction of P815 cells and quantitated based on phosphate as described (30), or were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. After evaporation of organic solvents and solubilization in DOC/TBS, synthetic lipids were mixed in a ratio of 98:2, dimyristoylphosphatidylcholine (DMPC):cholesterol.

Components were incorporated onto the beads by mixing H-2 antigen, lipid, and the 5-μm beads in DOC/TBS and dialyzing at 4°C for 36 to 48 hr to remove the detergent. Unless otherwise noted, the ratios used were 6 μg H-2K^k or 12 μg H-2^d or H-2^b per 10^7 beads and 5 nmol lipid per 10^7 beads, with 2 to 20 × 10^6 beads in 0.75 ml buffer. All added lipid associates with the beads even when 20 times this amount is added (23). When H-2 antigen is added above this level, the fraction associated with the beads decreases slightly in a reproducible fashion. Dialysis was against 0.6 liter TBS in a sterile tissue culture flask containing SM-2 biobeads (Bio-Rad, Richmond, CA; prepared according to the manufacturer's directions and used at 1 gm/ml sample being dialyzed) as a detergent absorbent. The flask was placed on a rotating platform (Roto Torque Rotator, Cole Palmer, Chicago, IL) to keep the 5-μm beads in suspension during the entire period of dialysis. After 24 hr of dialysis, 5 mM $CaCl_2$ was added to the dialysis buffer. Dialysis tubing (Spectra/Por 2 membranes, 12,000 to 14,000 MWCO; Fischer Scientific, Phoenix, AZ) was treated for 1 min in boiling water and was closed with clips (Spectra/Por closures; Fischer) after sample addition in order to completely exclude air bubbles from the bag. Complete exclusion of air is necessary to keep beads and lipid from accumulating at the air-fluid interface. After dialysis, the dialysis bag was cut open, and the beads were removed, washed three times in sterile medium, and stored at 4°C until added to culture.

The beads are well behaved in serum-containing medium and can be vortexed, transferred by plastic pipet, and collected by brief (1 to 2 min) low-speed centrifugation without losses, and can be counted by using a hemacytometer. When handled in protein-free buffer, such as in the papain cleavage experiments below, it is important to prevent exposure of the particles to an air-fluid interface. Thus, vortex mixing and complete removal of fluid after centrifugation are avoided.

Papain cleavage. Exposure of incorporated antigen at the bead surface was assessed by susceptibility to papain cleavage. For cleavage experiments, the beads were harvested without added serum, washed three times in TBS, and divided into aliquots of 7 × 10^5 beads (10,000 cpm ^{125}I-H-2K^k, 0.42 μg H-2K^k) for treatment with 0.33 U/sample soluble papain (type IV from papaya latex; Sigma), 1 U/sample papain immobilized on carboxymethylcellulose beads (Sigma), or no enzyme. The immobilized enzyme was washed three times in TBS before preactivation. Enzyme was preactivated for 30 min at 37°C with 50 mM cysteine, and 5 mM cysteine was present during the cleavage. After cleavage, beads were pelleted by centrifugation, and the ^{125}I-H-2K^k radioactivity in the supernatant fluid and pellets was determined. Cleavage for 90 min was found to release 80% as much radioactivity as was solubilized by overnight treatment.

Preparation of liposomes. Purified H-2K^k was incorporated into unilamellar liposomes by mixing with extracted cellular lipid (1 μg H-2K^k/20 nmol lipid) in DOC/TBS followed by dialysis to remove the detergent as described (30) except that liposomes were not harvested by centrifugation. After dialysis at 4°C against TBS for 24 hr, and 10 mM HEPES, 5 mM $CaCl_2$, 0.14 M NaCl, pH 7.9 for 12 hr, the stock solution of liposomes was used directly (23).

In vitro CTL generation and ^{51}Cr-release assay. Alloantigen-bearing beads, liposomes, or adherent cell-depleted allogeneic splenocytes were added to responder splenocytes from CD2F_1 mice primed 2 to 6 mo previously by i.p. injection of 2 × 10^7 RDM-4 (H-2K^k) cells and were cultured in 2 ml of medium with 7 × 10^6 responder cells/well (Linbro, New Haven, CT) at 37°C and 5% CO_2 for H-2^d anti-H-2K^k responses. For H-2K^k anti-H-2^d responses, responder splenocytes were from AKR/J mice primed 2 to 6 mo previously by i.p. injection of 2 × 10^7 P815 (H-2^d) cells. For H-2^b anti-H-2K^k responses, responder splenocytes were from C57BL/6 mice primed 2 to 6 mo previously by i.p. injection of 2 × 10^7 RDM-4 cells. Depletion of adherent cells was performed by incubating for 90 min at 10^7/ml and 5 ml/60-mm tissue culture-treated dish, followed by overnight culture of the nonadherent cells, and irradiation (3000 R) immediately before use as stimulators. An optimal concentration of an $(NH_4)_2SO_4$ fraction of supernatant fluid from rat spleen cells cultured with concanavalin A (Con A supernatant), prepared as described (31), was added at 22 hr of culture, unless otherwise indicated. All experiments included control cultures lacking alloantigen. The culture medium used was RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 50 μM 2-mercaptoethanol, and 5 mg gentamicin/dl.

After 5 days, each culture was assayed for cytotoxicity at several effector to target cell ratios by triplicate determination of the ^{51}Cr

released in 4 hr from 10^4 labeled RDM-4 or P815 target cells incubated at 37°C and 5% CO_2. The percent specific ^{51}Cr release = 100 × (experimental − spontaneous ^{51}Cr release)/(total released by 0.5% DOC − spontaneous ^{51}Cr release). Spontaneous release was less than 15% for all experiments. Lytic units provide an estimate of the relative number of effector CTL in a population (32). One lytic unit is defined as the number of cultured cells required to lyse 50% of the target cells in the 4-hr ^{51}Cr-release assay and are reported as lytic units per 10^6 cells.

Anti-Lyt-2 mAb inhibition of effector CTL generation was studied by using the 53-6.7 mAb (26). Culture wells having 7×10^6 responder splenocytes received either the 53-6.7 mAb or a control mAb; they were then incubated for 60 min at room temperature. Alloantigen-bearing stimulators were then added, followed by incubation at 37°C and 5% CO_2 for 5 days. An optimal amount of Con A supernatant was added as described above. Dose-response titrations showed 0.45 μg/culture well of 53.6-7 to maximally inhibit spleen cell stimulator-induced responses (data not shown), and this amount of 53-6.7 and control antibody was used in all experiments. This same amount of antibody was found not to block lysis when present during a chromium-release assay (see below). Thus, blocking by antibody added at the initiation of culture reflects blocking of recognition and triggering of precursor CTL, and not blocking of effector cell activity by antibody that might remain after 5 days of culture and washes before the lytic assay.

Anti-Lyt-2 mAb inhibition of effector CTL was performed by addition of the antibody to the ^{51}Cr-release assay with the use of 10^4 labeled RDM-4 target cells. After 5 days, effector CTL cultures were harvested and incubated for 45 min at room temperature in microtiter plates at 2×10^5 to 7.8×10^2 cells/well (in triplicate) with no antibody or with 10 μg mAb 53-6.7/ml. Labeled target cells were then added, and ^{51}Cr release was assessed after 4 hr at 37°C and 5% CO_2 as described above. The data are reported as lytic units per 10^6 cells.

Fluorescent staining and FACS analysis. Antigen-bearing beads and allogeneic spleen cells were prepared for FACS analysis exactly as those prepared for use in tissue culture (see above). Before analysis they were washed twice in sorter medium containing Hanks' balanced salt solution, 0.1% bovine serum albumin (Sigma; RIA grade), and 0.1% NaN_3, without phenol red or bicarbonate, pH 7.2. Samples were then split into aliquots for incubation with either anti-H-2K^k mAb (11-4.1) or an irrelevant anti-NP idiotype mAb (R3-I). Both antibodies are γ_{2a} subtype. Incubation for 30 min at 4°C with 5 μg mAb and 5×10^5 beads or cells was followed by three washes. Staining was then performed by using 0.5 μg/sample of a FITC-conjugated goat anti-murine γ_{2a} antisera for 30 min at 4°C. Antibodies were clarified by centrifugation in an airfuge for 5 min at 100,000 × G and 40°C immediately before use. Analyses were performed after three final washes on a Becton Dickenson FACS 440 with the use of an argon laser operating at 400 mW power and 488 nM. Fluorescence emission was collected by using a four-decade logarithmic amplifier. Data collection and analysis was performed on a Digital Electronics Corp. VAX 11/730 with DESK (copyright, Stanford University) software.

Phase-contrast and scanning transmission electron microscopy (STEM). Effector CTL cultures resulting from 5-day stimulation with 1.5×10^6 pseudocytes, as described above, were washed three times by centrifugation in culture medium, were resuspended, and were viewed microscopically. Phase-contrast observations were performed at a magnification of 400×, and photography was done by using Kodak Tri-X film. Alternatively, cells and beads were washed three times in serum-free phosphate-buffered saline, pH 7.2, and were allowed to settle by gravity onto glass coverslips coated with 1 mg/ml poly-L-lysine. Coverslips were then washed with phosphate-buffered saline, and the samples fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 20 to 30 min. After a brief buffer rinse, samples were postfixed in 1% OsO_4 for 20 min, rinsed with buffer, treated with a saturated solution of tannic acid for 5 min, rinsed again, and further fixed with 1% OsO_4. After a graded series of ethanol washes from 50 to 100% (5 min each), samples were placed in liquid freon 113 and were dried by substitution from freon to liquid CO_2, to dry CO_2 in a Polaron 3000 critical point dryer. Dried coverslips were coated by using a gold-palladium (80:20) source in an E5100 Polaron sputter coater. Samples were scanned by using a pointed filament in the secondary mode of a Hitachi H-600 scanning transmission electron microscope (33).

RESULTS

Stimulation of CTL responses by antigen-bearing beads (pseudocytes) is specific. As a solid support for the antigen-bearing membranes, 5-μm silica-based spherical particles, covalently modified with C18 alkyl chains, were used. Mixing of purified H-2 antigen, lipid, and the particles in deoxycholate, followed by dialysis to remove the detergent, results in controlled and reproducible incorporation of the H-2 antigen and lipid onto the beads (23). The resulting preparation consists of a fine suspension of individual beads that can be washed in sterile tissue culture medium and handled like cells. The size of the beads and the expression of H-2 antigen on their surface suggested that they might provide an extremely effective substrate for the study of CTL recognition (23) and examination of antigen density effects.

Recognition of H-2 alloantigens by resting spleen cells leads to proliferation of precursor CTL and their differentiation into effector CTL capable of lysing target cells that bear the stimulating antigen. Results from previous work have shown that in vitro generation of this response is dependent on helper T cell production of lymphokines (31), but that this dependence can be eliminated by addition of supernatants from mitogen-stimulated rat lymphocyte cultures (Con A supernatant). In this case, the magnitude of the lytic response that develops during 5 days in culture depends only on the effectiveness of alloantigen recognition by precursor CTL (31). The response to H-2 antigen on pseudocytes was therefore assessed by measuring their ability to stimulate effector CTL generation in cultures containing optimal amounts of Con A supernatant.

Class I antigen on beads is recognized specifically and without nonspecific mitogenic effects (Fig. 1). In double-reciprocal experiments, beads bearing H-2^k or H-2^d antigens were examined as stimulators of H-2^d anti-H-2^k and H-2^k anti-H-2^d responses. When splenocytes from CD2F$_1$ (H-2^d) mice, previously immunized with RDM-4 (H-2^k) cells, were cultured for 5 days in the presence of beads bearing H-2K^k antigen, effector CTL were generated that lysed RDM-4 but not P815 (H-2^d) target cells (Fig. 1*A* and *B*). Culture of the same cells with beads bearing H-2^d antigen or without beads resulted in no response with either tumor cell target used (Fig. 1*A* and *B*). Reciprocally, only H-2^d beads stimulated a response by AKR/J (H-2^k) spleen cells previously immunized to P815 (H-2^d), and the resulting effectors were specific for target cells bearing the appropriate H-2^d alloantigen (Fig. 1*C* and *D*). Spleen cells from C57BL/6 (H-2^b) mice, previously primed to RDM-4, were also stimulated by only H-2^k-bearing beads, and not by H-2^d- or H-2^b-bearing beads (data not shown).

Pseudocytes stimulate precursor CTL as efficiently as spleen cells but are unable to induce lymphokine production by helper T cells. Quantitative comparison showed that in the presence of added Con A supernatant, equal numbers of antigen-bearing beads or allogeneic spleen cells stimulated strong and comparable H-2^d anti-H-2^k responses, whereas no cytolytic response was obtained in the absence of alloantigen (Table I). The response to H-2 antigen on the 5-μm beads was more efficient than that to antigen on small (<0.1-μm) unilamellar liposomes in the presence of added factors, whereas the response to antigen on liposomes was greater than that to bead-bourne antigen in the absence of factors (Table I). In the absence of added factors, generation of effector CTL is limited by accessory cell presentation of antigen to helper T cells and subsequent in vitro generation of helper factors (31). H-2K^k-bearing liposomes stimulate a

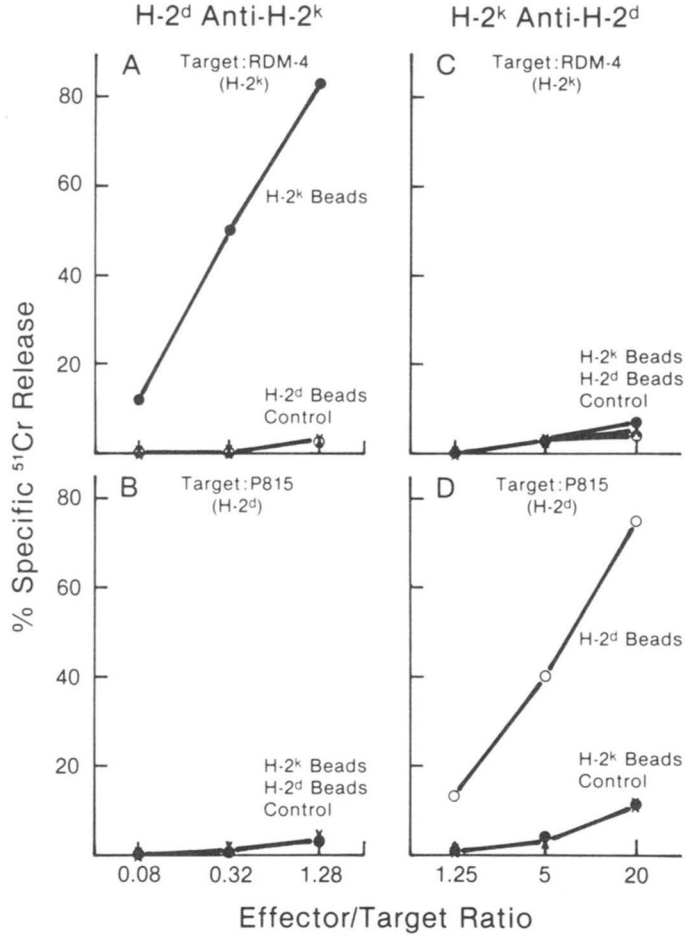

Figure 1. CTL stimulation by pseudocytes is specific. Splenocytes (*A*, *B*) from $CD2F_1$ ($H\text{-}2^d$) mice immunized 2 to 6 mo previously with RDM-4 cells ($H\text{-}2^k$), or (*C*, *D*) from AKR/J ($H\text{-}2^k$) mice immunized 2 to 6 mo previously with P815 cells ($H\text{-}2^d$), were cultured with antigen-bearing beads and Con A supernatant, as described in *Materials and Methods*. Antigens used were: 10^6 $H\text{-}2K^k$-bearing beads/culture (●), 10^6 $H\text{-}2^d$-bearing beads/culture (○), or no antigen (×). After 5 days the cultures were assayed for effector CTL generation by measuring the chromium release from ^{51}Cr-labeled (*A*, *C*) RDM-4 and (*B*, *D*) P815 target cells. Data are expressed as percent specific ^{51}Cr release at varying effector to target ratios.

TABLE I

Generation of CTL in the presence or absence of added lymphokines[a]

Antigen[b]	Response (lytic units)	
	+Con A Sn	−Con A Sn
Medium	<10	<10
Pseudocytes	680	50
Liposomes	270	110
Spleen Cells	670	500

[a] Responder spleen cells from CD_2F_1 ($H\text{-}2^d$) mice, primed 2 to 6 mo previously with RDM-4 ($H\text{-}2^k$) cells, were placed in culture with the indicated antigen in the presence or absence of Con A supernatant (Con A Sn), and lytic activity was assayed 5 days later by ^{51}Cr release from radiolabeled RDM-4 target cells. Data are expressed as lytic units per 10^6 cells.

[b] Antigens: $H\text{-}2K^k$-bearing pseudocytes were used at 9×10^5 beads/culture and bore 0.5 μg of H-2 antigen. Liposomes were used at 0.5 μg $H\text{-}2K^k$/culture. Spleen cells from A/J ($H\text{-}2K^k/D^d$) were adherent cell depleted, irradiated (3000 R), and used at 9×10^5 cells/culture.

significant response under these conditions because the alloantigen on liposomes can be taken up by accessory cells and can induce Ia-dependent helper T cell production of lymphokines (31). In contrast, the strong dependence of pseudocyte-stimulated responses on addition of exogenous helper factors indicates that bead-bourne antigen is stable and not available for accessory cell immune presentation.

The 5-μm antigen-bearing pseudocytes can be readily seen by light microscopy and are easily distinguished from cells (Fig. 2). When cultures stimulated with antigen-bearing beads are examined after 5 days, clusters of proliferating cells on beads and bead-lymphocyte conjugates are seen (Fig. 2*A* and *B*). A more detailed study of the antigen dependence of these interactions (23) has shown that conjugates are observed only rarely when beads bear syngeneic or irrelevant third-party Class I proteins or no surface H-2 antigen. Furthermore, addition of antibody specific for the H-2 antigen on the beads reversed conjugates, whereas treatment with an irrelevant antibody of the same subclass had no effect (23).

Increased amounts of H-2 antigen on pseudocytes increases CTL stimulation. Results from previous work have shown that the ability of alloantigen-bearing beads to stimulate generation of CTL was essentially the same whether extracted cellular lipids or synthetic lipids (DMPC/cholesterol) were used to coat the beads by dialysis, and that stimulation was unchanged over a wide range of incorporated lipid levels (23). In contrast, stimulation is dramatically effected by the level of H-2 antigen incorporated onto the beads (Fig. 3). Pseudocytes made by using 1.2 μg $H\text{-}2K^k/10^7$ beads stimulated almost no response by primed $CD2F_1$ ($H\text{-}2^d$) spleen cells, regardless of the number of beads added to culture, whereas beads made by using 6 μg antigen/10^7 beads stimulated a strong response.

The dramatic difference in the response stimulated by the beads made with higher levels of H-2 antigen is not explained by differences in the total amount of antigen added to cultures. Addition of 0.15 μg H-2 antigen to culture on 125×10^4 beads made with 1.2 μg antigen/10^7 beads stimulated almost no response, whereas the same total amount of antigen on 25×10^4 beads made with 6 μg antigen/10^7 beads resulted in a strong response (Fig. 3). These results suggested that recognition of antigen on the beads was dependent on the density of H-2 protein on the bead surface. Experiments were therefore done to determine the relationship between the amount of H-2 antigen mixed with the beads before dialysis and the density of antigen on the surface of the resultant pseudocyte preparation.

Controlled variation and quantitation of pseudocyte

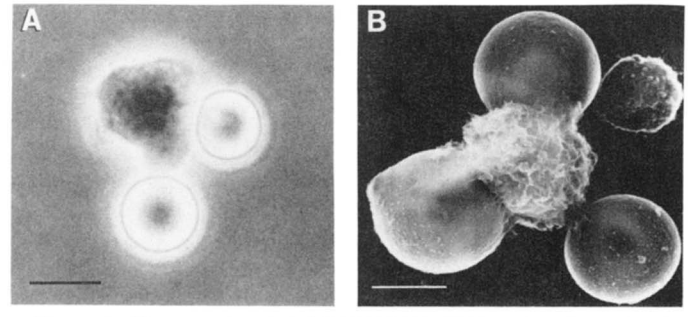

Figure 2. Phase-contrast and STEM photomicrographs of pseudocyte-cell conjugates. Splenocytes from $CD2F_1$ ($H\text{-}2^d$) mice immunized 2 to 6 mo earlier with RDM-4 ($H\text{-}2^k$) cells were cultured for 5 days with $H\text{-}2K^k$-bearing beads and Con A supernatant and were examined. The cultures containing both cells and beads were split into aliquots and (*A*) were washed with serum-containing culture medium and were viewed at 400× under phase-contrast illumination or (*B*) were washed with serum-free phosphate-buffered saline and were allowed to settle on poly-L-lysine-treated coverslips for visualization by STEM as described in *Materials and Methods*. The bars represent 5 μm.

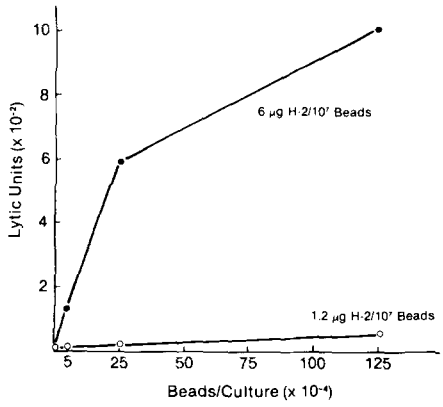

Figure 3. CTL stimulation by pseudocytes bearing low or high amounts of H-2 antigen. Splenocytes from $CD2F_1$ ($H\text{-}2^d$) mice immunized 2 to 6 mo previously with RDM-4 ($H\text{-}2^k$) cells were cultured in the presence of Con A supernatant and increasing numbers of alloantigen-bearing beads as described in *Materials and Methods*. After 5 days effector CTL generation was assessed by measuring the chromium release from ^{51}Cr-labeled RDM-4 cells. Beads bore either low amounts of $H\text{-}2K^k$ (1.2 μg/10^7 beads) (○) or high amounts of $H\text{-}2K^k$ (6 μg/10^7 beads) (●). Data are shown as lytic units per 10^6 cells.

TABLE II

H-2 antigen incorporation onto beads and release by papain treatment[a]

Sample	H-2 Antigen Added (μg/10^7 beads)[b]	H-2 Antigen Bound (%)[c]	H-2 Released by Papain (%)	
			Soluble	Immobilized
1	1.2	97	38	37
2	2.4	95	40	37
3	6.0	92	42	36
4	6.0	94	41	37

[a] The indicated amount of $H\text{-}2K^k$ was mixed with 5 nmol lipid and 10^7 5-μm beads in DOC/TBS, and the preparation was dialyzed as described in *Materials and Methods*.

[b] Lipids used were: 1–3, cellular lipids extracted from P815 cells; 4, synthetic lipids (98:2, DMPC:cholesterol).

[c] Incorporation and release of H-2 antigen was monitored by including $^{125}I\text{-}H\text{-}2K^k$ of known specific activity during coating of the beads. Papain cleavage was performed as described in *Materials and Methods*.

antigen surface density. Because essentially all added H-2 antigen was incorporated onto the beads upon dialysis to remove the detergent, the level of H-2 antigen incorporated per bead could be readily varied by changing the amount of Class I protein mixed with a constant number of beads before dialysis (Table II). Surface exposure of the antigen incorporated onto the beads, as demonstrated by its cleavage by papain, was about 40% whether low or high amounts of H-2 protein were present on the beads (Table II). Thus, an increasing amount of H-2 antigen was accessible at the bead surface as an increasing amount of antigen was incorporated onto the beads. In five separate experiments, the amount of H-2 antigen incorporated varied by less than 7% at any given dose of H-2 antigen per bead (data not shown). It was possible, therefore, to construct beads with chosen amounts of antigen exposed on the surface, using either synthetic or cellular lipids.

Similar amounts of H-2 antigen were released by cleavage with either soluble papain or papain immobilized on a solid support, and when either cellular lipids or synthetic lipids were used (Table II). Cleavage by the immobilized enzyme suggested that incorporated antigen was likely to be readily available for cellular recognition. The uncoated 5-μm beads used in these experiments had pores 50 to 110 angstroms in diameter; since H-2 molecules are about 5 Å in diameter, it seems likely that the fraction of bead-bourne H-2 antigen that was inaccessible to papain was located within these pores. The smooth and uniform surface appearance of beads coated with lipid and H-2 antigen when visualized by STEM (Fig. 2*B*) suggests that dialysis probably results in formation of a relatively continuous membrane on the beads (23).

FACS analysis after indirect immunofluorescent staining was done to determine if antigen on the bead surface was serologically active, and to confirm that the density of antigen on the beads was being varied in a controlled manner. Although the beads were found to have a significant level of autofluorescence (Fig. 4*A*, peak 1), specific staining by anti-$H\text{-}2K^k$ mAb and an FITC-conjugated second antibody was easily seen (Fig. 4*A*, peaks 2–4). The specificity of the staining was demonstrated in control experiments done by using no first antibody, by using an irrelevant first mAb of the same subclass, or by using beads coated with lipid but lacking H-2 antigen (Table III, samples 7–9). As expected, the greater the amount of antigen incorporated onto the beads by dialysis, the

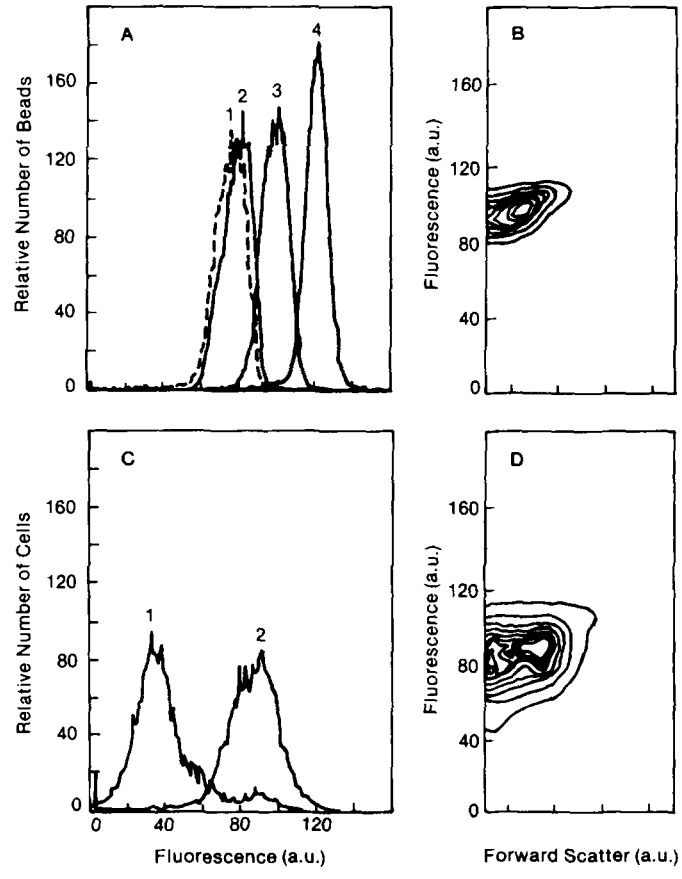

Figure 4. FACS analysis of pseudocyte and splenocyte size and H-2 antigen surface density. Pseudocytes formed by dialysis with different amounts of H-2 antigen, and adherent cell-depleted A/J ($H\text{-}2^a$) splenocytes, were prepared for FACS analyses by indirect immunofluorescent staining with anti-$H\text{-}2K^k$ mAb (11-4.1) or an irrelevant first mAb (R3-I), as described in *Materials and Methods*. Data are presented as histograms of particle number vs fluorescence in arbitrary units (a.u.): (*A*) unstained beads bearing 3.6 μg $H\text{-}2K^k/10^7$ beads (peak 1, – – – –), or beads stained with 11-4.1 mAb and bearing 3.6 μg (peak 2), 6.0 μg (peak 3) or 9.6 μg (peak 4) $H\text{-}2K^k$ antigen per 10^7 beads; and (*C*) A/J splenocytes stained with R3-I (peak 1), or 11-4.1 (peak 2). Forward light scatter (a.u.) is shown vs fluorescence (a.u.): (*B*) beads stained with 11-4.1 and bearing 6 μg $H\text{-}2K^k/10^7$ beads; or (*D*) splenocytes stained with 11-4.1. Fluorescence by beads stained with R3-I was the same as that for beads bearing no H-2 antigen and stained with the $H\text{-}2K^k$-specific mAb (Table III).

greater the level of anti-H-2K^k mAb bound to the beads (Fig. 4*A* and Table III). Examination of the stained beads by fluorescence microscopy showed the fluorochrome to be uniformly distributed over the beads (23) at all surface density levels (data not shown), indicating that the antigen was not highly aggregated.

Adherent cell-depleted spleen cells from A/J mice were also examined by FACS analysis performed with the same reagents (Fig. 4C and *D*). Measurement of forward light scatter indicated that the antigen-bearing beads were more uniform in size than spleen cells (Fig. 4*B* and *D*). It was also apparent that the beads were more homogeneous than cells with respect to surface density of antigen, as shown by comparison of peak widths (Fig. 4*A* and C). Furthermore, bead fluorescence intensity varied as a function of bead size (Fig. 4*B*), as expected for a population of uniform antigen density; but this was not the case for cells, which had widely varying densities of H-2 antigen on cells of every size (Fig. 4*D*). Thus, not only can surface antigen density on beads be precisely varied, but the range of densities on individual beads in a preparation is much narrower than that found on normal spleen cells.

Effect of membrane antigen density on stimulation of CTL responses. Having established that surface antigen density could be varied in a controlled manner, we performed experiments to determine the effect of antigen density on the ability of the beads to stimulate a CTL response. For clarity, antigen densities will be referred to hereafter on a relative scale, with the arbitrary definition that beads made with 1.2 μg H-2 antigen/10^7 beads express a 1× relative density. Such a relative scale is valid, since a constant fraction of incorporated H-2 protein is exposed on the bead surface over the range of antigen levels under study (Table II).

TABLE III

FACS analysis of pseudocyte H-2 antigen surface density[a]

Sample	H-2 Added[b] (μg)	H-2 Bound[c] (a.u.)	Antibodies[d] Anti-H-2	Anti-NP Id	Anti-mIg (FITC)	Fluorescence channel number mean (coef var)[e]
Beads						
1	1.2	1x	+	−	+	100.6 (11)
2	2.4	2x	+	−	+	103.6 (10)
3	3.6	3x	+	−	+	107.6 (09)
4	4.8	4x	+	−	+	115.7 (09)
5	6.0	5x	+	−	+	123.3 (08)
6	12.0	8x	+	−	+	150.1 (06)
7	0.0	0x	−	+	+	99.0 (11)
8	4.8	4x	−	+	+	99.5 (11)
9	6.0	5x	−	+	+	98.1 (11)
10	3.6	3x	−	−	−	93.3 (10)
Spleen cells						
11	—	—	+	−	+	107.3 (18)
12	—	—	−	+	+	50.4 (49)
13	—	—	−	−	+	50.2 (48)

[a] Pseudocytes were prepared by dialysis as described in *Materials and Methods* and were studied by FACS analysis after indirect immunofluorescent staining. A/J spleen cells (H-2^a) were stained for FACS analysis after depletion of adherent cells and overnight incubation, as described in *Materials and Methods*.

[b] The indicated amount of H-2K^k was mixed with 5 nmol lipid and 10^7 beads in DOC/TBS, and the preparation was dialyzed as described in *Materials and Methods*.

[c] The relative amount of H-2 antigen associated with the beads was assessed by incorporation of ^{125}I-H-2K^k and is reported in arbitrary units (a.u.), which define the amount of antigen bound by beads in sample 1 as 1x.

[d] For FACS analysis a sample was incubated with either the 11-4.1 anti-H-2K^k mAb (anti-H-2), samples 1–6 and 11, or an irrelevant mAb (anti-NP Id) of the same subtype, samples 7–9, and 12, followed by staining with an FITC-conjugated goat anti-murine γ_{2a} antiserum (FITC-anti-mIg), or incubated in the absence of all antibodies, sample 10, as described in *Materials and Methods*.

[e] The mean fluorescence channel number (log scale) and the coefficient of variation (coef var) in the mean are reported.

When bead-supported membranes bearing various densities of H-2 antigen on their surfaces were used to stimulate cultures of CD2F$_1$ (H-2^d) mice previously immunized to RDM-4 (H-2^k) cells, the response was found to be highly dependent on H-2 antigen density on the stimulating beads (Fig. 5). A relative H-2 antigen density of 1× was completely insufficient to generate a measurable response from this alloantigen-primed population despite the presence of an optimal number of beads per culture (see below) and added Con A supernatant. At a relative H-2 antigen density just fourfold higher, the response was maximal. The response plateaued at a relative H-2 antigen density of 4×, above which further increase in H-2 density no longer augmented the response.

The fact that response as a function of antigen density exhibits a threshood and a rapid rise to a plateau might be explained in one of two ways. All of the precursor CTL might be capable of recognition and response to a given antigen density, but require that increasing numbers of beads be present as antigen density decreases. Alternatively, increased response to increasing antigen density might represent the recruitment of a greater fraction of the potentially responsive precursor CTL in the population, and reflect their individual antigen density requirements for recognition and triggering. Comparison of the response to relative density 2× vs 5× antigen-bearing beads as a function of particle number added to culture (Fig. 6) rules out the first of these possibilities. Although addition of 10^6 beads/culture stimulates a maximal response in both cases, the response to a relative H-2 antigen density of 2× reached a plateau level significantly below that seen to 5× beads. Thus, some of the precursor CTL are not able to respond to the 2× beads regardless of the number of beads (amount of antigen) added to culture.

In addition to differing in the plateau level of response they stimulate, 2× vs 5× beads differ with respect to dose-response effects at limiting bead numbers. Thus, in the experiment shown (Fig. 6), addition of 10^5 2× density beads/culture induced generation of only a 50-lytic unit response, whereas addition of the same number of 5×

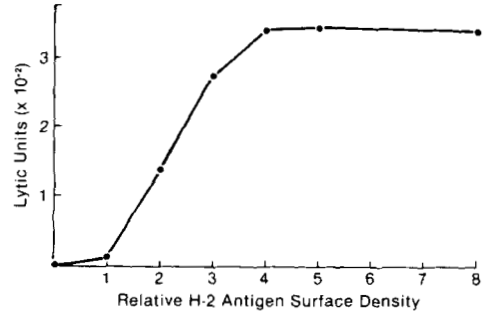

Figure 5. The effect of increasing H-2 antigen surface density on CTL stimulation by pseudocytes. Splenocytes from CD2F$_1$ (H-2^d) mice immunized 2 to 6 mo previously with RDM-4 (H-2^k) cells were cultured with 10^6 beads/culture and Con A supernatant. Beads were made with trace amounts of ^{125}I-H-2K^k antigen so that relative incorporation of antigen could be assessed by cpm. A relative H-2 antigen incorporation of 1.2 μg H-2K^k/10^7 beads is defined as 1×. Cytotoxicity was assessed after 5 days of culture by using ^{51}Cr-labeled RDM-4 target cells, and the results are expressed as lytic units per 10^6 cells.

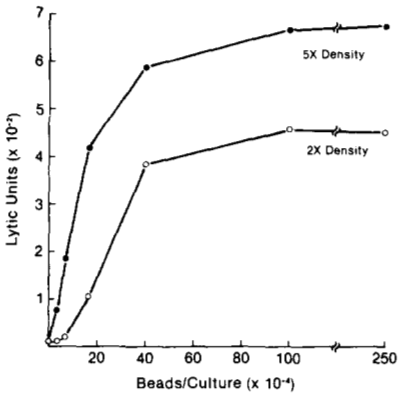

Figure 6. CTL stimulation by increasing numbers of pseudocytes bearing low or high surface densities of H-2 antigen. Splenocytes from $CD2F_1$ (H-2^d) mice, immunized 2 to 6 mo previously with RDM-4 (H-2^k) cells, were cultured with increasing numbers of alloantigen-bearing beads and Con A supernatant, as described in *Materials and Methods*. After 5 days, effector CTL generation was assessed by measuring the chromium release from ^{51}Cr-labeled RDM-4 cells. Beads bore either (○) 2× (2.4 μg H-$2K^k/10^7$ beads) or (●) 5× (6 μg H-$2K^k/10^7$ beads) relative surface densities of alloantigen. Data are shown as lytic units per 10^6 cells.

TABLE IV

The effect of anti-Lyt-2 antibody on effector CTL generation[a]

Expt.	Antigen[b]	Response (lytic units)		
		No mAb	+Anti-Lyt-2	(% Inhib)
1	2x H-2 bead	310	30	(90)
	5x H-2 bead	870	526	(40)
2	2x H-2 bead	217	17	(92)
	5x H-2 bead	488	227	(55)
3	2x H-2 bead	333	15	(96)
	5x H-2 bead	630	270	(57)
4	5x H-2 bead	455	267	(41)
	Spleen cells	667	351	(47)

[a] Responder cells were splenocytes from $CD2F_1$ (H-2^d) mice, primed 2 to 6 mo previously with RDM-4 (H-2^k) cells, preincubated in the presence or absence of anti-Lyt-2 mAb, and cultured with the indicated antigen and Con A supernatant. After 5 days, lytic activity was assessed by ^{51}Cr release from radiolabeled RDM-4 target cells, as described in *Materials and Methods*. Percent inhibition was calculated by dividing the response in the presence of antibody by the response in its absence. Data are reported as lytic units per 10^6 cells.

[b] Antigens: H-$2K^k$-bearing pseudocytes were used at 10^6 beads/culture and were made with either 2.4 μg H-$2K^k/10^7$ beads (2x H-2 Bead) or 6 μg H-$2K^k/10^7$ beads (5x H-2 Bead), as described in *Materials and Methods*. A/J (H-$2K^k/D^d$) splenocytes were adherent cell depleted, irradiated (3000 R), and used at 10^6 cells per culture (spleen cells), as described in *Materials and Methods*.

density beads resulted in generation of a response of 260 lytic units. This probably reflects a higher precursor CTL frequency of cells able to respond to 5x rather than 2× density beads, and thus the greater likelihood that a given precursor cell that comes into contact with a bead at low input numbers will be capable of response. It may also result from a lower percent of successfully triggered responses, despite bead recognition, when antigen valency is low (2×).

Relationship between antigen density and susceptibility of CTL responses to blockade by anti-Lyt-2 antibody. It has been suggested that the Lyt-2/3 complex functions to facilitate the interaction of CTL with target cells, particularly when the strength of their association is weak (19, 20). The ability to vary the density of stimulating alloantigen on the beads (and thus presumably control their degree of interaction with antigen-specific cells), made it possible to examine the relationship between antigen density and susceptibility to anti-Lyt-2 mAb blockade at the level of precursor CTL recognition. Cultures of $CD2F_1$ (H-2^d) mice previously immunized with RDM-4 (H-2^k) cells were preincubated in the presence or absence of anti-Lyt-2 mAb and then cultured with pseudocytes bearing relative H-$2K^k$ antigen densities of 2× or 5×. The generation of effector CTL was assessed after 5 days. Anti-Lyt-2 mAb was titrated in initial experiments to determine the level needed for maximal blockade. This level of antibody (0.25 to 0.45 μg mAb/7 × 10^6 splenocytes) did not block effector CTL function when added directly to a ^{51}Cr-release assay. Thus, effects seen in these experiments reflect blocking of generation of the response, and not blocking of effector CTL function at the end of the culture period.

Anti-Lyt-2 mAb inhibited the response of precursor CTL to H-2 antigen-bearing pseudocytes (Table IV). The degree of inhibition varied inversely with the surface density of alloantigen on the beads. Thus, not only was the level of response to low (2×) density beads lower than that to high (5×) density beads, as expected, but in the presence of anti-Lyt-2 mAb the response to low density beads was virtually abolished (Table IV). In contrast, the response to high density beads was inhibited by only 40 to 57%, the same range observed for blockade of spleen cell-stimulated responses (Table IV) in six separate experiments (data not shown). Blocking in these experiments was specific for anti-Lyt-2, as addition of the same amount of anti-θ mAb had no blocking effect on the response to low or high density beads or to splenocytes (data not shown). Thus, increased antigen surface density results in decreased susceptibility to blocking by anti-Lyt-2 mAb at the level of recognition by precursor CTL.

The relationship between stimulating antigen density and the susceptibility of the resulting effector CTL to anti-Lyt-2 mAb blockade was also examined. If precursor CTL that are able to respond to 2× density beads do so because they can interact strongly with low valency antigen, and if such effector CTL are therefore less dependent on the function of Lyt-2/3 proteins, then it would be predicted that target cell lysis by a population resulting from stimulation with 2× density beads would be less susceptible to mAb blockade than a population resulting from stimulation with 5× density beads. This in fact was found. In two separate experiments, target cell lysis by CTL generated in response to low density beads was inhibited by only 20 to 24% by addition of 10 μg/ml of anti-Lyt-2 antibody (see *Materials and Methods*). In contrast, lysis of the same targets by CTL generated in response to high density beads was inhibited by 50 to 55% by the same level of antibody and lysis by CTL generated in response to allogeneic spleen cells was inhibited by 70 to 80% (addition of anti-θ mAb resulted in less than 20% inhibition with any of the CTL populations). Thus, the progeny of precursor CTL capable of response to low valency antigen are effector CTL that appear less dependent on Lyt-2/3 complex function.

DISCUSSION

The results presented in this paper demonstrate that the response of alloantigen-reactive precursor CTL is determined by the density of Class I molecules on antigen-bearing membrane surfaces. Recognition and response to alloantigen was assessed by measuring the development of lytic activity after in vitro stimulation of resting spleen cells from immune animals. To stimulate

a response, a minimum threshold surface density of antigen was required. At a relative H-2 antigen density just fourfold higher than threshold, the response was maximal. Blockade of precursor and effector CTL responses by anti-Lyt-2 antibody was also examined with respect to antigen density dependence. Direct evidence was provided for a reciprocal relationship between antigen density and susceptibility of precursor CTL stimulation to blockade. Thus, the response to low density antigen was highly susceptible to antibody inhibition, whereas the response to high density antigen was not. The results also demonstrated that those effector CTL resulting from stimulation with low density antigen were less susceptible to anti-Lyt-2 mAb blockade than CTL generated to high density antigen, presumably because they are capable of interacting with target cells effectively even under conditions where the valency of interaction is limited.

Previous efforts to analyze the role of membrane antigen density in CTL recogition have involved manipulation of intact antigen-bearing cells to alter surface density (3–8). Interpretation of such studies is complicated, first, by the fact that the surface membrane of a cell is dynamic and can rapidly change in composition, and second, by the fact that manipulation of the cells can change parameters in addition to surface antigen density, and thereby alter CTL recognition (4, 11–14). The approach described in this report eliminates these problems, and was made possible by the development of a novel artificial membrane system in which transmembrane proteins and lipid are incorporated onto the surfaces of cell-size particles.

The artificial membranes were prepared by dialysis to remove detergent from a mixture of purified antigen, lipid, and 5-μm silica beads covalently modified with C_{18} alkyl chains. The H-2 antigen-bearing beads, termed pseudocytes (23), stimulated generation of secondary CTL responses with the same specificity (Fig. 1) and efficiency (Table I) as alloantigen-bearing spleen cells, provided that lymphokines (Con A supernatant) were added to the cultures (Table I). Subcellular antigen in the form of liposomes can be taken up by accessory cells in culture to a sufficient extent to allow processing and presentation of their alloantigen to helper T cells and thereby stimulate endogenous lymphokine production (31). In contrast, bead-bourne antigen is very ineffective in generating this helper response, as evidenced by the more than 10-fold increase in response upon addition of exogenous lymphokines (Table I). These results strongly support the conclusion that precursor CTL recognize Class I antigen on the surface of the beads (23), as does the observation of lymphocyte-bead conjugates in cultures containing effector CTL (Fig. 2), which are antigen-specific (23).

Pseudocytes made with low or high amounts of incorporated H-2 antigen were dramatically different in their ability to stimulate effector CTL generation (Fig. 3), suggesting a critical role for antigen surface density in precursor CTL stimulation. Analysis of the surface exposure of antigen on pseudocytes formed by using different amounts of Class I protein showed that antigen density on the beads could be readily controlled. Indeed, at all levels of incorporation, about 40% of the H-2 protein was accessible to both soluble papain, and papain immobilized on a solid support (Table II). This conclusion was confirmed, and serological activity of the surface H-2 antigen was demonstrated, by FACS analysis (Table III). Further, bead preparations were found to be considerably more homogeneous than spleen cells with respect to both antigen density and size (Figure IV). Thus, pseudocytes having a surface antigen density of choice could be prepared in a controlled and reproducible manner. The susceptibility of pseudocyte surface H-2 antigen to papain cleavage, its binding by anti-H-2 antibody, and its recognition by precursor CTL, all indicate that the antigen maintains its native conformation and is exposed on the lipid-coated surface of the bead in a manner similar to its exposure on a cell surface.

Having established that antigen density on the bead surfaces could be reproducibly varied, it was possible to determine how density affected precursor CTL triggering. Recognition of Class I alloantigen by precursor cells, as measured by the subsequent generation of effector CTL, was found to be highly dependent on the H-2 antigen density on the stimulating beads (Fig. 5). Response required a threshold density of surface H-2 antigen, and increasing the density just fourfold above this threshold resulted in stimulation of a maximal response (Fig. 5). Neither the threshold effect nor the marked dependence on density over a relatively narrow range had been previously seen for T cell recognition and triggering. This may be due, in part, to the fact that normal allogeneic cells are heterogeneous with respect to antigen surface density, and this heterogeneity is also likely to be present after treatment of cells to alter H-2 antigen surface density.

The maximal level of response to large numbers of low density beads was considerably below that reached upon stimulation with high density beads (Fig. 6); that is, low response to low surface antigen density on individual beads could not be overcome by increasing the total amount of antigen added to culture. This result very strongly suggests that some precursor CTL in a population can recognize and be triggered by interaction with either low or high density antigen, whereas other precursor CTL must interact with high density antigen to be triggered. These differences in density requirements may reflect differences in a number of parameters, including affinity of the antigen-specific receptor, density of these receptors on the T cell, and presence and number of accessory proteins on the T cell.

The notion that T cell response involves interaction of multiple low affinity receptors (34), and requires highly multivalent antigen for avid binding and for triggering of responses, is supported by cases where binding of nominal antigen to T cells has been reported (35, 36). Evidence for the importance of multivalent interaction in alloantigen recognition has also been obtained in studies to compare CTL stimulation by Class I antigen in protein micelles, in liposomes of varying sizes (37), and on cell-size beads of the type described here (23). The requirement for a high antigen density on the cell-size beads (Fig. 5), comparable to that on normal spleen cells, to stimulate a maximal response provides strong additional support for the notion that extensive membrane receptor and ligand cross-linking may be required for effective binding and transmembrane signaling to occur. This requirement is likely to have important biological consequences in controlling the immune response. For example, low H-2 Class I antigen expression has been found

to correlate with transplant survival of allogeneic tumorigenic cells, whereas high antigen expressors were eliminated (9, 10). In addition, interferon has been reported to increase susceptibility of virus-infected cells to lysis by CTL (38). One of the cellular effects of interferon is to increase levels of Class I antigen expression (8). It is notable in this regard that CTL release interferons upon interaction with target cells (39) and that poorly recognized target cells induce higher levels of interferon production by CTL than do cells that are readily lysed (40).

The ability to control the surface density of alloantigen on the beads also made it possible to examine the relationship between antigen density and the function of the Lyt-2/3 accessory protein complex of precursor and effector CTL. Stimulation of precursor CTL by beads bearing suboptimal densities of surface H-2 antigen was highly susceptible to anti-Lyt-2 mAb blockade (Table IV). Antibody virtually abolished the response to these low density beads, whereas the response to high density beads or splenocytes was reduced by only 40 to 57% (Table IV). Conversely, when precursor CTL were allowed to respond to beads bearing low densities of H-2 antigen in the absence of blocking antibody, the resultant effector CTL were found to be more resistant to blockade than effectors generated in response to high density beads. Presumably, the CTL resulting from recognition of low density beads are the progeny of precursor CTL that require only low valency interactions for response. Effector CTL populations resulting from stimulation with beads bearing high densities of antigen would include these highly responsive cells as well as cells that could not respond to low antigen density. This latter population might be expected to be more strongly dependent on highly multivalent CTL-target cell interaction, and therefore more susceptible to blockade by anti-Lyt-2 mAb.

These results are consistent with a reciprocal relationship between susceptibility to anti-Lyt-2 mAb blocking and responsiveness of the T cell to antigen, and may reflect the strength of interactions between the CTL and target cell ligands. Anti-Lyt-2 antibodies have been shown to interfere in the recognition-adhesion step of antigen-bearing target cell lysis (20, 41). It has been suggested (19, 20), therefore, that Lyt-2/3 molecules are involved in strengthening CTL-target adhesion, and that this role is particularly important when the strength of the interaction with antigen is low. In this model, T cell response reflects the overall "avidity" of interaction between the CTL and antigen-bearing structure, and this "avidity" reflects a summation of the various receptor-ligand affinities. Thus, variations in response arise from either differences in T cell receptor affinity (18) or density of antigen (and thus multivalency of interaction) (7, 8), or both. Such a model is also consistent with recent studies of helper T cell responses. Cloned T cell hybridomas that were more susceptible to anti-L3T4 mAb blockade of MHC-restricted stimulation also required higher stimulating antigen dose to induce response (42).

These considerations, together with the observed correlation between Lyt-2/3 expression and Class I-restricted function (21), have led to the proposal that the Lyt-2/3 accessory complex may function to strengthen interaction via binding to conserved determinants on Class I MHC proteins (21), a suggestion made even more attractive by the recent demonstration that the gene encoding Lyt-2 is a member of the immunoglobulin supergene family (43, 44). However, other experiments have provided strong evidence to argue that cross-linking of Lyt-2/3 molecules by antibody results in delivery of a negative signal to the T cell, and thus blocking of response by anti-Lyt-2/3 mAb need not necessarily involve blocking of an interaction with a ligand (22). Our results do not distinguish between these two possibilities. They do, however, argue very strongly that if Lyt-2/3 interacts with a ligand on the antigen-bearing cell, then that ligand must be Class I MHC protein, since it is the only protein present on the beads.

The cell-size artificial membranes described in this report are readily prepared with well-defined and easily quantitated components and can be used like antigen-bearing cells in functional studies. They have many advantages over the use of metabolically active cells or previously described artificial membranes. Liposomes added to culture are readily taken up by accessory cells (31), have the potential to fuse into the membranes of other cells, and are recognized in a limited manner because of their small size (23, 37). Artificial membranes supported on glass slides (45) and large (100-μm) glass beads (46, 47) are limited with respect to ability to characterize the membranes, manipulate the antigen-bearing structures, and by requirements for large amounts of antigen. For example, 100-μm glass beads (46) are poorly suited to microscopic examination of cellular interaction at 8000 times the volume of cells or the beads used in this study and require 400 times as much material to reach a similar density of antigen per bead. The very small amounts of antigen required to prepare large numbers of cell-size beads (<6 $\mu g/10^7$) will make this approach useful for studying surface proteins that cannot be readily isolated in as large amounts as native Class I and II antigens. For example, MHC-encoded antigens altered by exon shuffling or site-directed mutagenesis and expressed in cells might be purified, and their recognition could be studied in the absence of confounding effects such as levels of expression on the transfected cell or the contributions of host cell surface proteins.

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