Journal of Supramolecular Structure
and Cellular Biochemistry 16:121–131 (1981)
Cellular Recognition 143–153

Lymphocyte Recognition of H-2 Antigen in Liposomes

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Liposomes containing purified H-2K^k will specifically stimulate generation of a secondary allogeneic cytolytic T lymphocyte response. Effective recognition was found to depend on the structure of the liposomes. Including detergent-insoluble plasma membrane matrix during formation resulted in liposomes having two- to fourfold more activity than those prepared using just lipid and H-2.

Key words: H-2 antigen, liposomes, plasma membrane matrix, cytolytic T lymphocytes, cell–liposome interaction, H-2 recognition

Cytolytic T lymphocytes (CTL) specific for allogeneic cells recognize the H-2K or D cell surface glycoproteins on these cells. Recognition leads to binding and lysis of the allogeneic target cells. It has proved to be difficult to study this recognition event at the molecular level (see Balk and Mescher, this volume). More success has been achieved in studying antigen-specific recognition leading to generation of a secondary CTL response. Spleen cells from mice previously immunized with allogeneic cells include a population of pre-CTL which, when placed in culture with irradiated allogeneic stimulator cells, differentiate to become active CTL. This event also requires specific recognition of the H-2K or -D antigens.

With the demonstration that purified plasma membranes [1] and solubilized, reconstituted membranes [2, 3] containing H-2 antigens can be used instead of whole cells to stimulate generation of a secondary CTL response, it has become possible to study the cellular and molecular requirements for this response in more detail. The use of monoclonal antibody affinity chromatography has made it possible to obtain relatively large amounts of purified H-2K and -D antigens [4, 5], thus allowing incorporation of these proteins into artificial membranes and study of their recognition by lymphocytes. Initial studies using purified H-2K^k in liposomes showed that these preparations stimulated a CTL response very inefficiently in comparison to plasma membranes containing an equivalent amount of H-2 antigen. It was found, however, that much more effective stimulation could be obtained if an actin-containing matrix isolated from tumor cell plasma membranes was included during formation of the liposomes [6]. This matrix contains actin and several other proteins and is localized at the inner face of the plasma membrane where it appears to form a membrane skeleton [7] (Herrmann and Mescher, manuscript in preparation). When isolated from purified plasma mem-

Received April 10, 1981; accepted June 2, 1981.

0275-3723/81/1602-0121$03.50

branes, it remains largely in the form of closed structures having the same size distribution as the original membrane vesicles.

The reproducibly high stimulating activity of these matrix-containing liposomes has made it possible to study in more detail the cellular requirements for generation of a secondary in vitro CTL response. It has become apparent in the past several years that Ly1$^+$ T helper cells can augment alloreactive CTL responses [8, 9], and that splenic adherent cells and macrophages play a role in CTL activation [10, 11]. Recent studies have shown that an effective response to H-2 in liposomes requires two antigen recognition events. One of these appears to involve direct recognition of the alloantigen by the Ly2$^+$ pre-CTL [12] (Herrmann et al, manuscript in preparation). The second involves uptake and processing of the antigen by adherent cells and presentation to Ly1$^+$ helper T cells (T_H) [12–14]. These T_H cells then produce a factor which, together with alloantigen recognition, triggers the pre-CTL to differentiate. The requirement for adherent cells and Ly1$^+$ cells can be bypassed by the addition of T cell growth factor to the cultures [12].

We have previously shown that liposome formation in the presence of the isolated membrane matrix results in incorporation of H-2 and the matrix into the same structures, and that these are more effective in specifically stimulating an in vitro CTL response than liposomes containing only H-2 and lipid [6]. This report describes experiments to characterize further the matrix-containing liposomes and to study their recognition by lymphocytes.

METHODS

Mice and Cell Lines

C57B1/6(B6) (H-2^b), (Balb/c × DBA/2)F1 (CD2F1) (H-2^d), AKR (H-2^k), A/J (H-2^a), and (AKR × DBA/2)F1 (AKD2) (H-2$^{d\,k}$) mice, 6–12 weeks of age, were purchased from Jackson Laboratories, Bar Harbor, Maine, or Cumberland View Farms, Clinton, Tennessee. Tumor cell lines P815 (H-2^d), RDM-4 (H-2^k), YAC (H-2^a), and EL4 (H-2^b) were maintained in mice of the corresponding H-2 haplotype. RDM-4 cells for use in large scale H-2K^k purification were grown in AKD2 mice.

Preparation of Plasma Membranes, Matrix, H-2K^k, and Lipid

Tumor cell plasma membranes were purified as previously described [1], and the plasma membrane matrix was isolated as previously described [7]. Briefly, purified membranes were incubated for 20 min at 4°C in 0.5% Triton X-100, PBES (10 mM sodium phosphate containing Earl's salts, pH 7) at a concentration of 1 mg/ml. The insoluble matrix was then pelleted by centrifugation for 45 min at 100,000g, washed once with PBES, and suspended in 0.5% deoxycholate (DOC) in 10 mM Tris buffer, 0.14 M NaCl, pH8 (TBS) for use in liposome preparation. Approximately 20% of the total membrane protein is recovered in the detergent-insoluble matrix fraction [7].

H-2K^k was purified from RDM-4 tumor cell lysates using a monoclonal antibody affinity column as previously described [4]. ^{125}I-labeled H-2K^k was purified in the same way from cells labeled by lactoperoxidase-catalyzed iodination. The labeled protein was mixed with unlabeled H-2K^k, and the specific activity was determined by assaying protein content.

In experiments done to examine the CTL stimulating activity of H-2 in liposomes, the amount of antigen used is expressed in cell equivalents (ceq) (ie, the number of cells from which that amount of antigen was derived) to facilitate comparison to stimulation by plasma membranes. Thus 10^5 ceq is equal to 0.5 μg of RDM-4 membrane or 0.015 μg of purified H-2K^k.

Lipids used for preparation of liposomes were obtained from P815 tumor cells by chloroform:methanol (2:1) extraction. Extract was washed with 0.3 volumes of water, dried under an N_2 stream, and resuspended in 0.5% DOC-TBS.

Preparation of Liposomes

Liposomes were prepared by mixing H-2K^k and lipid in 0.5% DOC-TBS. If used, matrix was also added to the mixture. The relative amounts of each component used varied and are indicated in the figure and table legends. The mixtures were then dialyzed at 4°C for 24 hr against TBS containing 0.2 mM phenylmethyl sulfonyl fluoride (PMSF) followed by an additional 24-hr dialysis against TBS containing 5 mM $CaCl_2$. The resulting liposomes were harvested by centrifugation at 100,000g for 45 min; the supernatant was discarded, and the liposome pellet was resuspended in buffer or culture medium.

CTL Stimulation

The CTL stimulating activity of membranes and liposomes was assessed by placing the antigen into culture along with spleen cells from previously immunized mice. After 5 days in culture the cells were assayed for their ability to lyse ^{51}Cr-labeled target cells bearing the appropriate H-2 antigen. Antigen was added to 2-ml cultures containing 7×10^6 spleen cells from CD2F1 mice immunized 1–3 months previously with 2×10^7 YAC tumor cells [1]. After 5 days in culture the cytotoxicity was assayed by a standard 4-hr ^{51}Cr release assay [15] using 2×10^4 ^{51}Cr-labeled target cells. The percent specific release and lytic units were calculated as previously described [1]. One lytic unit (LU) is defined as the number of lymphocytes required to give 50% lysis of 1×10^4 target cells in 4 hours. Results are expressed as LU/10^6 spleen cells. Spontaneous release of ^{51}Cr from the target cells was less than 24% for all experiments.

Analytical

Protein was determined by the method of Lowry et al [16] or the orthophthalaldehyde method of Butcher and Lowry [17] using bovine serum albumin as the standard. Lipid concentration was based on organic phosphate content [18]. 5′-Nucleotidase activity was assayed as described by Avruch and Wallach [19].

RESULTS

Characterization of H-2-Containing Liposomes

Preparation of liposomes by detergent dialysis of a mixture of H-2 and lipids as described in Methods resulted in incorporation of about 30% of the added H-2K^k into vesicles that can be sedimented by centrifugation at 100,000g. When detergent-insoluble matrix is included, about 50% of the H-2 is incorporated. Similar results were found when dimyristoylphosphatidylcholine was used instead of lipids extracted from tumor cells.

Liposomes prepared using lipid and H-2 at a ratio of 20 nmol phospholipid:10^7 ceq H-2K^k (approximately 11:1, wt:wt) were found to have a density of about 1.02–1.03 g/cm^3 on sucrose gradients (Fig. 1A), similar to that found for liposomes containing purified HLA and phospholipid made at a similar ratio [20]. Including matrix during liposome formation resulted in formation of vesicles of higher density and a more heterogeneous distribution on a sucrose gradient (Fig. 1B), and increasing the ratio of matrix protein to H-2 and lipid further increases the density of the vesicles (Fig. 1C). The ratio of lipid to matrix protein used to form the vesicles of Figure 1B is approximately the same as the ratio of these components in intact membranes (Mescher and Jose, manuscript submitted). The distribution of H-2 and 5′-nucleotidase, a matrix component [7], across the gradients (Fig. 1B, C) strongly suggests that the antigen and matrix are incorporated into the same vesicle structures. This has been further confirmed by examination of the vesicle components by SDS-gel electrophoresis [6].

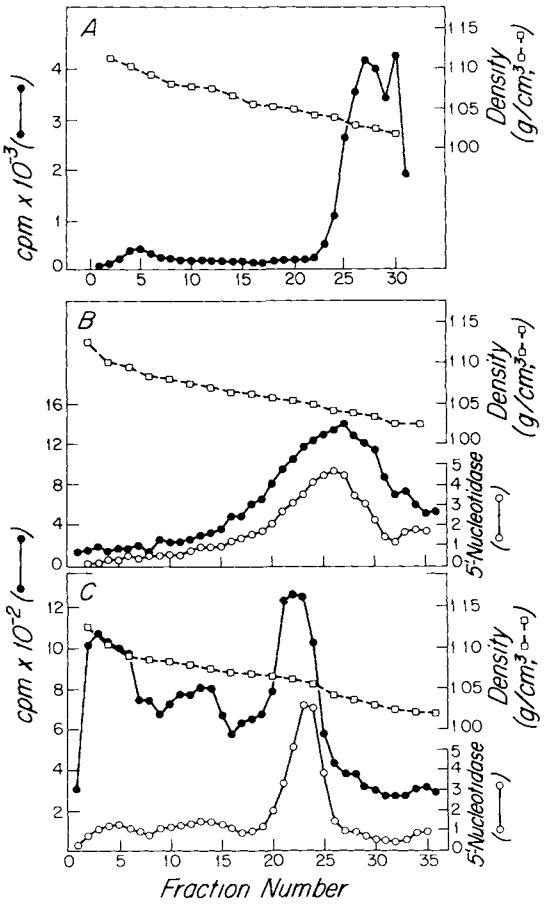

Fig. 1. Sucrose density gradient centrifugation of H-2K^k-containing liposomes. Centrifugation was done as previously described [6]. Liposomes were prepared from: A) 200 nmol lipid and 15 μg H-2K^k; B) 200 nmol lipid, 15 μg H-2K^k and 50 μg detergent-insoluble matrix; C) 200 nmol lipid, 15 μg H-2K^k and 250 μg matrix. —●—● ^{125}I-H-2K^k, —○—○— 5′-nucleotidase, --□--□-- density of sucrose.

Accessibility of the H-2 antigen on the liposome surface is likely to be an important parameter affecting lymphocyte recognition. Papain treatment of liposomes prepared with and without matrix demonstrated that about 70–85% of the antigen was accessible to the enzyme in both cases (Fig. 2). Addition of 0.5% TX-100 prior to papain treatment resulted in degradation of >97% of the H-2. It has previously been shown that incorporation of MHC antigens into liposomes by detergent dialysis results in preferential orientation on the outer surface [20–24]. This appears to be true also for liposomes made in the presence of matrix. The antigen that was not completely degraded by papain was found to have a molecular weight about 4,000 daltons lower than that of native antigen (Fig. 2). A protease (trypsin, papain)-sensitive cleavage site is present in the C-terminal (cytoplasmic)

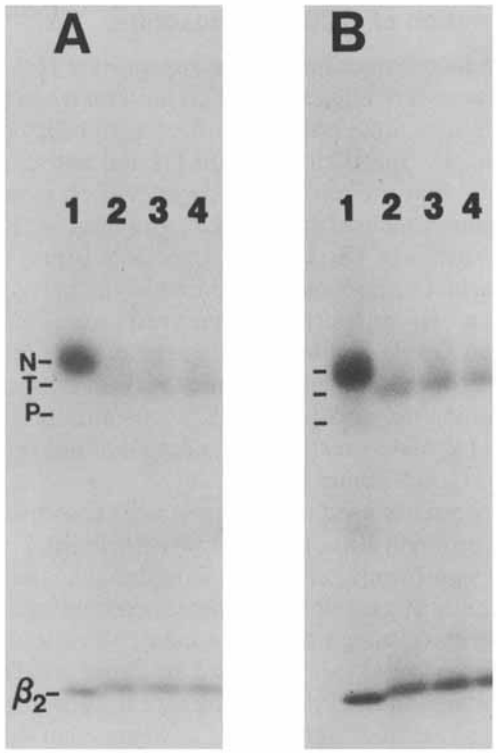

Fig. 2. Removal of liposome-associated H-2K^k with papain. Lipid-H-2 liposomes were prepared from 150 nmol lipid and 15 μg H-2K^k. Matrix-containing liposomes were prepared from 150 nmol lipid, 15 μg H-2K^k, and 125 μg matrix. ^{125}I-H-2K^k was included in both preparations. Liposomes were incubated at 37°C with 3.8 μg/ml papain (activated by incubation for 30 min at 37°C with 10 mM cysteine) in TBS with 2.5 mM cysteine. Aliquots were removed at varying times and made 20 mM in iodoacetamide to inactivate the papain. Samples were run on 5–15% polyacrylamide gradient gels [6], protein visualized by Coomassie blue staining and autoradiography (shown) and the H-2 quantitated by cutting out the bands and measuring the ^{125}I radioactivity. A) H-2K^k + lipid liposomes: 1) 0 m, 100% remaining; 2) 30 m, 15% remaining; 3) 60 m, 14% remaining; 4) 120 m, 13% remaining. B) H-2K^k + lipid + matrix liposomes: 1) 0 m, 100% remaining; 2) 30 m, 27% remaining; 3) 60 m, 27% remaining; 4) 120 m, 26% remaining. N, position of native heavy chain. T, position of trypsin cleavage product (see text). P, position of papain cleavage product.

region of the H-2 heavy chain (Herrman and Mescher, manuscript in preparation), and appearance of the lower molecular weight product upon treatment of the liposomes suggests that the antigen present on the inside of the liposome is inserted in the bilayer in a transmembrane orientation with the C-terminal region exposed on the surface.

Isolation of the detergent-insoluble matrix from plasma membrane vesicles results in material that is present as closed structures having the same size distribution as the original vesicles [7]. Liposomes prepared from just lipid and H-2 have the appearance of small unilamellar vesicles (Fig. 3A), whereas liposomes prepared in the presence of matrix are larger and more irregular in shape (Fig. 3B). These observations, together with the results of density gradient centrifugation and SDS-gel electrophoresis [6], suggest that both the lipid and H-2 become associated with the matrix upon removal of detergent by dialysis.

Lymphocyte Recognition of H-2K^k in Liposomes

Purified plasma membranes having the appropriate H-2 antigens will stimulate generation of a secondary allogeneic CTL response when placed in culture with spleen cells from mice previously immunized with cells bearing the same allogeneic H-2 antigen [1]. Specificity controls [1] and antisera-blocking studies [25] have demonstrated that the response is dependent on recognition of the H-2 antigen. When liposomes prepared from H-2K^k and lipid were examined for their ability to stimulate a specific CTL response, they were found to be relatively ineffective (Table I). In contrast, liposomes prepared in the presence of matrix were much more effective, a two- to fourfold greater response resulting from a given amount of H-2 antigen (Table I). Furthermore, the maximum response obtainable with matrix-containing liposomes was two- to threefold greater than that obtained with liposomes containing only lipid and H-2. Liposomes prepared using just matrix and lipid (no H-2) have no stimulating activity, and they do not enhance the response to lipid–H-2 liposomes [6].

The lipid–H-2 liposomes used in the experiment shown in Table I were made using a phospholipid:protein ratio of 11:1(wt:wt). Increasing or decreasing this ratio twofold did not significantly affect the stimulating activity of the liposomes. Beyond these limits the activity decreased. Matrix-containing liposomes were optimally active when prepared using a matrix protein:H-2 ratio of from 5 to 20 (Table II). Activity decreased at ratios below 5 or above 20. These results suggest that the density of antigen on the liposome surface is important for effective recognition by lymphocytes. It does not appear, however, that differences in antigen density on the surface can account for the differences in stimulating activity of the two types of liposome.

Experiments were also done to examine the possibility that lipid–H-2 liposomes might be less effective owing to inhibitory or toxic effects. This did not appear to be the case, as adding both lipid–H-2 liposomes and matrix-containing liposomes to the same responder cells simply resulted in an additive response (Table III). Experiments done to examine specificity of stimulation also ruled out the possibility that matrix-containing liposomes might be nonspecifically mitogenic. Spleen cells from CD2F1 (H-2^d) mice previously primed with H-2^b cells responded in vitro to EL4 (H-2^b) membranes but not to RDM-4 (H-2K^k) membranes or liposomes containing H-2K^k and matrix (Table IV). Similarly, spleen cells from

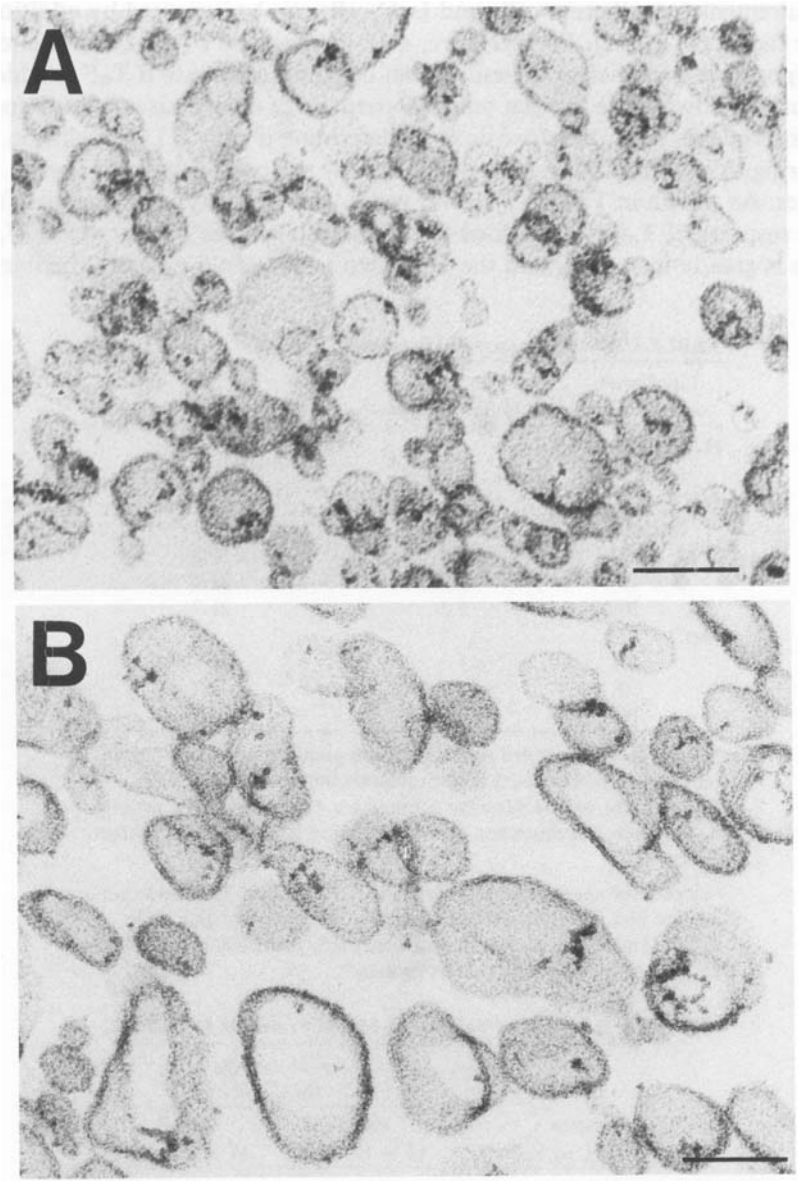

Fig 3 Thin-section electron micrographs of liposomes prepared from A) lipid and H-2K^k, and B) lipid, H-2K^k, and matrix. Bar represents 0 1 μ.

AKR (H-2K^k) mice previously immunized with H-2^d cells responded to P815 (H-2^d) membranes but not H-2K^k liposomes containing matrix (not shown).

Recent studies have shown that stimulation of a secondary response by liposomes requires two signals: 1) antigen recognition by the pre-CTL; and 2) adherent cell uptake, "processing," and presentation of the antigen to Ly1$^+$ T cells [12–14]. The possibility existed, therefore, that the greater effectiveness of matrix-containing liposomes might result from more effective uptake by adherent cells.

The requirement for adherent cells and Lyl$^+$ cells can be bypassed by addition of T helper factor (T_HF) [12]. Furthermore, a 12-hr exposure of the responder cells to H-2K^k liposomes is sufficient to result in an optimum response if T_HF is added to the cultures following the antigen pulse (Herrmann et al, manuscript in preparation). Experiments were therefore done to determine if pre-CTL recognition of antigen during this 12-hr pulse was also affected by the composition of the liposomes. As shown in Table V, a 12-hr pulse with either type of liposome results in a low response if T_HF is not added to the cultures. In the presence of T_HF, the response is greatly increased, and the same two-to threefold greater effectiveness is

TABLE I. Dose Response to Liposomes*

Liposome[a] composition	H-2K^k + lipid	H-2K^k + lipid + matrix
H-2K^k added (ceq)	LU	LU
Exp 1		
2×10^4	1.0	1.3
2×10^5	1.7	5.8
2×10^6	5.9	21.1
10^7	7.3	21.7
Exp 2		
10^4	<1	2
10^5	2.9	5
10^6	6.1	14.3

*Liposomes were added to 2 ml cultures along with 7×10^6 spleen cells from CD2F1 (H-2^d) mice previously immunized with YAC (H-2^a) tumor cells. Cells were cultured for 5 days, and CTL activity was measured as described in Methods using RDM-4 (H-2K^k) target cells.

[a]Liposomes were prepared as described in Methods. Those containing just H-2 and lipid were made using 200 nmol lipid and 15 μg H-2K^k. Those that include matrix were made using 200 nmol lipid, 15 μg H-2K^k, and 150 μg matrix protein.

TABLE II. Effect of Varying the Matrix Protein to H-2 Ratio*

Ratio[a] (μg matrix/μg H-2K^k)	CTL activity (lytic units) Exp 1 (2×10^5 ceq)	Exp 2 (4×10^5 ceq)
No matrix	1.4	3.6
1.7	2.9	–
5	7.0	20.0
10	4.1	26.0
20	4.9	–
33	3.8	20.0
67	3.2	3.8

*CTL stimulating activity was assayed as described in Methods and Table I.

[a]Liposomes were prepared using mixtures containing 200 nmol lipid, 15 μg H-2K^k, and varying amounts of isolated matrix.

TABLE III. Effect of Mixing H-2K^k-Lipid Liposomes and Matrix-Containing Liposomes*

Antigen added[a]	CTL activity (% specific release) E:T 5	25	50
0	3	7	14
5 × 10^5 (H-2 + lipid)	3	15	20
5 × 10^5 (H-2 + lipid + matrix)	48	70	78
5 × 10^5 (H-2 + lipid)[b] + 5 × 10^5 (H-2 + lipid + matrix)	53	79	84

*CTL activity was assayed as described in Methods and Table 1. Results are expressed as % specific release at three different effector-to-target (E:T) ratios.

[a]Liposomes were prepared as described in Table I.

[b]Both types of liposomes were added to the same culture wells.

TABLE IV. Specificity of CTL Stimulation by H-2K^k in Liposomes*

Responder: CD2F1 (H-2^d) α EL4 (H-2^b)
Targets: EL4 (H-2^b)

Antigen[a]	CTL activity (% specific release) E:T 1	5
–	0	0
1 μg EL4 (H-2^b) membrane	18	58
5 μg EL4 (H-2^b) membrane	35	76
5 μg RDM-4 (H-2^k) membrane	0	7
10^5 ceq (H-2K^k + lipid + matrix)	0	0
10^6 ceq (H-2K^k + lipid + matrix)	0	1

*Antigen was added to 2 ml cultures along with 7 × 10^6 spleen cells from CD2F1 (H-2^d) mice previously immunized with EL4 (H-2^b) cells. Cells were cultured for 5 days, and CTL activity was measured as described in Methods using EL4 (H-2^b) target cells.

[a]Matrix-containing liposomes were prepared as described in Table I.

TABLE V. Comparison of CTL Stimulating Activity of Liposomes in the Presence of T_HF*

Immune spleen cells + antigen → 12 hr, 37°C → wash ↓ ↑ add T_HF → 4d → Assay CTL activity

Antigen	Addition	CTL activity (lytic units)
–	–	0.5
–	T_HF	1.4
2.5 × 10^5 (H-2K^k + lipid)	–	2.8
2.5 × 10^5 (H-2K^k + lipid)	T_HF	8.8
2.5 × 10^5 (H-2K^k + lipid + matrix)	–	3.6
2.5 × 10^5 (H-2K^k + lipid + matrix)	T_HF	24.8

*Spleen cells from previously immunized CD2F1 mice were mixed with antigen, incubated for 12 hr with mixing, washed to remove antigen, and placed in culture with or without T_HF. Preparation of T_HF has been described [12]. CTL activity was measured as described in Methods, using RDM-4 target cells.

seen with matrix-containing liposomes as is seen in continuous cultures. It therefore appears that more effective antigen presentation by matrix-containing liposomes occurs at the level of lymphocyte recognition, and is not simply due to enhanced uptake of these liposomes by adherent cells.

DISCUSSION

The transmembrane glycoproteins H-2 and HLA can readily be incorporated into lipid vesicles by detergent dialysis procedures to yield small unilamellar vesicles having the majority of the antigen exposed on the liposome surface [21–24]. Formation of liposomes by similar procedures in the presence of isolated plasma membrane matrix [7] results in formation of larger vesicles, which appear to have the matrix proteins, H-2K^k and lipid incorporated into the same structures. These liposomes also have about 75% of the incorporated antigen accessible on the surface.

H-2K^k liposomes made with matrix are more effective in specifically stimulating a CTL response than liposomes containing just lipid and H-2. A number of possible explanations for this difference have been ruled out. Density of H-2 on the liposome surface appears to affect recognition by lymphocytes, as the stimulating activity is affected by the ratio of H-2 to other components used for liposome formation. Density differences do not appear to account for the difference in effectiveness of the two types of liposome, however, since the difference is maintained when the ratios of components are optimized for both. Experiments also indicate that the difference in effectiveness cannot be accounted for by inhibitory or toxic effects of the liposomes containing only H-2 and lipid (Table III); nor can it be accounted for by nonspecific mitogenic activity of matrix-containing liposomes (Table IV). It also appears that the difference cannot be accounted for by enhanced uptake of matrix-containing liposomes by adherent cells, since a similar difference in effectiveness is seen in the presence of T_HF under conditions where adherent cells and Lyl$^+$ cells are not required (Table V).

The greater effectiveness of matrix-containing liposomes might result from the fact that the larger size and more irregular shape may allow for more highly multivalent interaction between antigen on the liposomes and receptors on the lymphocyte. A requirement for multivalent interaction being necessary to trigger the response is strongly suggested by the observations that the soluble, serologically active papain cleavage product of H-2 will not stimulate a response [26] (Herrmann and Mescher, unpublished results). Alternatively, the greater effectiveness could result from an interaction between the transmembrane H-2 and a component(s) of the matrix, which affects the display of the antigen on the liposome surface. Koch and Smith [27] have presented evidence suggesting that H-2 may interact with actin under some conditions. Both of these possible explanations are being investigated.

Understanding the basis for the greater effectiveness of matrix-containing liposomes should provide insight into the requirements for lymphocyte recognition of cell surface MHC antigens. Furthermore, the ability to prepare liposomes with reproducibly high stimulating activity allows the events occurring during generation of a response to be studied in greater detail. Cells can be exposed to antigen for defined periods of time, and the antigen can then be removed by differential

centrifugation, thus allowing pre-CTL recognition of antigen to be studied independently of events requiring adherent cells and Lyl$^+$ cells (Table V, Herrmann et al, manuscript in preparation). The use of radioactively labeled antigen will also make it possible to follow the fate of the antigen in culture, an approach that should prove useful in studying uptake, "processing," and presentation by adherent cells.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grant CA-14723. S.H.H. was supported by a Cancer Research Institute (New York) postdoctoral fellowship.

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