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THE ROLE OF SIALIC ACID
IN THE RECOGNITION OF H-2K\textsuperscript{b} ANTIGENS
BY CYTOTOXIC T CELLS

by

STEVE DAVID FIGARD

A Dissertation submitted to the
Department of Chemistry
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Approved:

Professor Directing Dissertation

[Signatures]

August, 1984
ABSTRACT

The H-2K^b major histocompatibility complex glycoprotein antigens from EL4 and from a carbohydrate-variant subline, WD1, were purified on an anti-H-2K^b (B8-24-3) monoclonal antibody affinity column. Previous studies have demonstrated that the cell-surface forms of the H-2 molecules from the WD1 variant line are complex but lack the terminal sialic acid residues. Each purified antigen was reconstituted along with purified cytoskeletal proteins into membrane vesicles of defined lipid composition, protein composition, and lipid/protein ratio.

A sensitive microassay was refined to detect the purified H-2K^b antigens in the liposomes. The assay utilizes the liposomes to inhibit specific conjugate formation between allogeneically primed (H-2^d anti-H-2^b) cytotoxic T cells and H-2^b target cells. As little as 5 ng of purified H-2K^b plus 23 ng cytoskeletal proteins in vesicles inhibited conjugate formation to 50% of the maximum inhibition observed. This inhibition was shown to be specific in two ways: 1) control vesicles containing purified H-2^k proteins inhibited conjugate formation but only at 5- to 7-fold higher H-2^k concentrations, and 2) the
same H-2K\textsuperscript{b}-containing vesicles did not inhibit nonspecific conjugate formation.

Liposomes containing H-2K\textsuperscript{b} from either EL4 or WD1 similarly inhibited specific conjugate formation between EL4 target cells and the allogeneically primed cytotoxic T cells. These results suggest that the presence or absence of the terminal sialic acid residues on the two N-asparagine-linked complex carbohydrates of H-2K\textsuperscript{b} did not affect the recognition step between the CTL and vesicles.
DEDICATION

This dissertation is dedicated to my wife, Renee Michelle, for her constant love, understanding, prayers, and support throughout my Ph.D. program; to my daughter, Kristin Helen, who saw less of her Dad than she wanted in her first two years; and to my mother and father, whose prayers and support have carried me through these years. May this Ph.D. be used to God's glory, and for the furtherance of His kingdom. Amen.
ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

ABTS, 2, 2'-azino-di-(3-ethylbenz-thiazaline sulfonic acid); BS, borate buffered saline; BSA, bovine serum albumin; b2-m, beta-two microglobulin; Con A, concanavalin A; CTL, cytotoxic T lymphocytes; CP, cytoskeletal proteins; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; EGTA, ethyleneglycol-bis-(beta-aminoethyl ether) N,N'-tetraacetate; FCS, heat-inactivated fetal calf serum; FDA, fluorescein diacetate; H-2^b, murine H-2 gene region of the b haplotype; H-2^k, murine H-2 gene region of the k haplotype; H-2^K, gene products of the H-2^K and H-2^D regions of the k haplotype; H-2^K^b, gene product of the H-2^K region of the b haplotype; H-2^K^k, gene product of the H-2^K region of the k haplotype; HRP-anti-rat-IgG, horseradish peroxidase-linked rabbit anti-rat IgG; HS, heat-inactivated horse serum; LcH, lentil lectin; LPA, Limulus polyphemus agglutinin; moAb(s), monoclonal antibody(ies); MHC, Major Histocompatibility Complex; Mr, relative molecular weight; NVS, normalized viability score; PBES, sodium phosphate buffer containing Earle's salts; PBS, phosphate buffered saline; PM, plasma membrane; PMSF, phenylmethylsulfonyl-fluoride; PS, penicillin-streptomycin; SA, sialic acid;
SBA, soybean agglutinin; SPM, serum-free medium; SDS, sodium dodecyl sulfate; t.c., tissue culture; TC, target cell; TM, tunicamycin; TNP, trinitrophenyl; Tris-HCl, Trizma Base; WGA, wheat germ agglutinin.
INTRODUCTION

Vertebrates possess a surveillance mechanism, called the immune system, that specifically recognizes and selectively eliminates pathogenic microorganisms, such as bacteria and viruses, and cancer cells. The immune mechanism operates on two different levels that provide distinct but overlapping protection. The humoral immune response defends primarily against the extracellular phases of infection by the secretion of antibodies, proteins that bind specifically to the foreign substance and initiate a variety of elimination responses. The cellular immune response is mediated by cells whose function is the recognition and lysis of fungi, parasites, virally infected cells, cancer cells, and foreign tissue.

The principal mediators of cellular immunity are the cytotoxic T lymphocytes (CTL). Our understanding of the mechanisms by which CTL recognize and lyse their targets is still very much on the phenomenological level. Evidence for at least three distinct phases of a lytic cycle for CTL has been compiled from kinetic analyses, observations of individual interacting CTL and target cells (TC), and studies of temperature and drug effects on the different steps.

The initial step, called conjugate formation, involves
the specific recognition and binding of the CTL to the TC. This binding is reversible (Balk & Mescher, 1981; Balk et al., 1981) and requires Mg\(^{2+}\) (Stulting & Berke, 1973), metabolic energy (Berke & Gabison, 1975; Todd, 1975; MacDonald & Koch 1977), and an active system of microfilaments (Plaut et al., 1973; Stulting et al., 1973; Golstein et al., 1978; MacLennan & Golstein, 1978; Ryser et al., 1982). It occurs within 1-2 min after contact (Martz, 1975), and the resulting CTL-TC conjugate is polyvalent, i.e., one CTL can bind more than one TC and vice versa (Berke et al., 1975). The optimum temperature for conjugate formation is 22\(^\circ\)C-37\(^\circ\)C, but conjugates will form at temperatures as low as 4\(^\circ\)C (Berke & Gabison, 1975).

The second step is sometimes called programming for lysis. Within 5-10 min after conjugate formation (Martz, 1975), an irreversible lethal hit is administered to the TC in a Ca\(^{2+}\)-dependent mechanism (Golstein & Smith, 1976; Golstein & Smith, 1977; Martz, 1977; MacLennan & Golstein, 1978), occurring optimally at 37\(^\circ\)C (Berke & Gabison, 1982). Interestingly, when one CTL binds more than one TC, the TC are lysed sequentially rather than simultaneously (Berke, 1980; Perelson & Bell, 1982).

Having programmed the TC for lysis, the CTL now disengages in the third, final step and recycles to other TC (Zagury, et al., 1975; Martz, 1976). The TC will lyse
anywhere between 10 min to 3 hr later (Zagury et al., 1975; Sanderson, 1976).

Compelling evidence indicates that the TC membrane antigens recognized as foreign by CTL are coded for by the major histocompatibility complex (MHC) (Berke & Fishelson, 1975; Schrader et al., 1975; Schrader & Edelman, 1976; Bevan & Hyman, 1977; Dennert & Hyman, 1977). This recognition can occur within two different contexts: allogeneic or syngeneic. In the allogeneic context, the specific collection of alleles comprising the MHC, called the haplotype, is different for the CTL and the TC. The MHC antigens of the TC are the direct antigenic stimulus for the CTL, and the CTL are, therefore, primed to recognize determinants on those specific antigens. This is the principal cause of allograft rejection in transplantation work.

In contrast, the haplotypes of the CTL and TC are identical in the syngeneic system. The antigenic stimulus is the introduction of other foreign surface molecules (e.g., viral glycoproteins or chemically-modified proteins) that are recognized in association with the MHC antigens. Primed syngeneic CTL, therefore, recognize a determinant comprised of both the MHC antigen and the other surface antigen. The exact nature of the association of the two antigens is still uncertain.
The MHC is designated H-2 in mice, and HLA in humans, and it is located on chromosome 17 in mice and chromosome 6 in humans. The classic transplantation antigens, H-2K and H-2D in mouse, HLA-A, and -B in humans, are also referred to as the serologically defined antigens (Bach et al., 1972) or the class I antigens (Klein, 1979). They have general tissue distributions, and our knowledge of their structure is at a more advanced state than that of other MHC-encoded products. Using intra-H-2 recombinants and mutants in mice, it has been shown that CTL recognize specificities coded by the H-2K and H-2D genes (Nabholz et al., 1974; Blanden et al., 1975; Shearer et al., 1975; McKenzie et al., 1977; Klein, 1978).

The class I MHC antigens are two-chain molecules formed by noncovalent interactions between the glycosylated heavy chain (Mr about 44,000-50,000) and beta-2 microglobulin (b2-m; Mr about 13,000) (Vitetta & Capra, 1978; Strominger et al., 1981). The heavy chain is the MHC gene product and spans the membrane bilayer with the antigenic N-terminal end directed towards the outside of the cell and the C-terminal end towards the cytoplasm (Walsh & Crumpton, 1977).

The entire primary sequence of the H-2K\textsuperscript{b} heavy chain is known (Coligan et al., 1981) and, together with ancillary data, has led to the following structural picture. The folded polypeptide chain has three external folding domains, each approximately 90-100 amino acids, defined from
exon-intron junctions (Steinmetz et al., 1981). These domains have been designated N, C1, and C2 (N is the first external domain, and C2 is the domain closest to the membrane) spanning amino acids 1-281. A hydrophobic membrane portion spans amino acids 282-308, and the intracellular C-terminus containing a cluster of basic amino acid residues spans amino acids 309-346. The C1 and C2 domains each contain one intra-chain disulfide linkage, and the C2 domain, a highly conserved region (Reyes et al., 1982), associates noncovalently with the nonpolymorphic β2-m (Nairn et al., 1980; Yokoyama & Nathenson, 1983). Most of the variability in H-2 molecules occurs in the regions 62-83 (in the N domain) and 95-121 (in the C1 domain) (Maloy & Coligan, 1982).

Studies using monoclonal antibodies (mcAb) against H-2 molecules have demonstrated that the antigenic determinants are concentrated in at least two spatially separated clusters on the H-2K^k heavy chain (Lemke et al., 1979). Using these same mcAbs to block CTL killing in a 51Cr-release assay, evidence has been reported suggesting that the same two clusters on the H-2K^k antigen are recognized by both allogeneic CTL and syngeneic trinitrophenyl (TNP)-specific CTL (Weyand et al., 1981). Additional antibody competition studies with a series of anti-H-2K^b mcAbs have shown that most of the antigenic determinants on mutant H-2K^b map to the N and C1 domains of
the molecule (Hammerling et al., 1981). Using hybrid H-2\textsuperscript{d} gene products expressed on mouse L cells after DNA-mediated gene transfer, Ozato et al. (1983) have reported that $^{51}\text{Cr}$-release and cold target inhibition of $^{51}\text{Cr}$-release occurs only when the $^{51}\text{Cr}$-labeled L cells express the N and C1 domains of the H-2 molecule to which the CTL are primed but not when the other domains are expressed. Thus, the polymorphic determinants recognized by alloreactive CTL were shown to be located primarily in the N domain, the C1 domain, or possibly in both. Similar conclusions were reached regarding H-2 recognition by syngeneic virus-specific CTL (Reiss et al., 1983). In a more recent work, Allen et al. (1984) constructed four hybrid class I genes in which exons were exchanged between the H-2K\textsuperscript{b} and H-2D\textsuperscript{b} genes and expressed the hybrid genes in mouse L cells, as above. Again, most CTL recognition sites and alloantigenic determinants are found in the N and C1 domains. In addition, they concluded that the CTL recognition sites and alloantigenic determinants are not influenced by interactions of those domains with the polymorphic regions of C2 but that they may be altered by interactions between N and C1. In one additional study, a dominant CTL recognition site was probably located in the C1 domain spanning residues 147-157 of the HLA-A2 molecule (Ware et al., 1983).
H-2K<sup>b</sup> and H-2D<sup>b</sup> have two N-asparagine-linked oligosaccharides in common at residues 86 and 176. H-2D<sup>b</sup> has an additional carbohydrate chain at residue 256 (Gilmer, 1982; Maloy & Coligan, 1982). Each oligosaccharide is comprised of 10-15 carbohydrate units (Tabas et al., 1978), depending on the subcellular source. The endoplasmic reticulum contains a high mannose form with a Mr of about 3000 (Gilmer, 1982). In contrast, the plasma membrane contains a complex carbohydrate form constructed of N-acetylglucosamine, mannose, galactose, fucose, and sialic acid (Gilmer, 1982). Using HPLC analysis, the structures of the two oligosaccharides on H-2K<sup>k</sup> have been shown to be complex and primarily biantennary (Sweidler et al., 1983; cf. Figure 1).

Although many, if not all, of the serologically defined specificities of the MHC antigens are on the polypeptide portion, little is known of the role the saccharide moieties might play in specific CTL-TC interactions. Given that the cell goes to great biochemical expense to put the oligosaccharide side chains onto the protein, the purpose for their presence is no trivial question.

Carbohydrates may modify the physicochemical properties of proteins by changing their hydrophobicity, electrical charge, mass, and size. The carbohydrate, however, rarely affects the biological activities of proteins, is not usually a prerequisite for protein secretion, and is not
Figure 1. Proposed structures for N-asparagine-linked oligosaccharides on H-2 antigens as found in the endoplasmic reticulum before any major carbohydrate processing (left), and in the plasma membrane (right).
usually antigenic. Notable exceptions to these generalizations can be found. In some systems glycosylation is necessary for cell-surface expression, and in others it is not (cf. references in Gilmer et al., 1984). Carbohydrates do serve as important recognition markers on soluble glycoproteins as well as on cell surfaces. (Glycoprotein structure and function is reviewed in Sharon and Lis, 1981)

It is interesting to note that the oligosaccharides on the H-2K\textsuperscript{b} antigens are located near the domain junctions of the heavy chain and that the highly conserved asn-86 oligosaccharide in particular is located between the two highly variable sequences (which are at 62-83 and 95-121). This positions the carbohydrate in the center of the two domains recognized by CTL and in between the two most probable regions for that recognition. There is, therefore, a strong potential for this oligosaccharide to modulate either the recognition of the target antigenic regions or the effector (i.e., lytic) function of the CTL by steric, electrostatic, and/or conformational effects induced by the sugars. CTL can distinguish conformational determinants induced by only one or two amino acid differences in H-2K\textsuperscript{b} molecules (Sherman, 1982).

One line of investigation into the effect of the carbohydrate in immune cell interactions uses tunicamycin (TM), an antibiotic which inhibits the en bloc transfer of
the high mannose carbohydrate to the polypeptide chain early in the glycosylation processing (Leavitt et al., 1977). TM treatment of target cells modestly inhibits the cytolysis of those target cells by alloimmune or by virus-specific, H-2-restricted CTL (Black et al., 1981; Harris et al., 1981). Pretreatment of allogeneic stimulator cells with TM prevents their induction of a primary blastogenic response by thymic lymphocytes (Hart, 1982).

These reports indicate an as yet undetermined role of the asparagine-linked carbohydrate in cellular recognition. However, the evidence is indirect, and some doubt can be raised because of the influence of TM on whole cell functions. Does it only inhibit glycosylation and nothing else? Does it influence, directly or indirectly, other cell functions involved in the recognition of the target cells by CTL? TM (via its effect on glycosylation?) has been shown to interfere with the insertion or function of cell-surface glycoproteins (Duksin & Bornstein, 1977) and to create defective transport of glucose, nucleotide sugars, uridine, and amino acid analogs (Olden et al., 1979; Yusuf et al., 1983). It decreases glucose metabolism (Olden et al., 1979), which is significant to the present discussion because glucose is required to obtain suspended conjugates (Shortman & Golstein, 1979). The studies cited above prove to be inconclusive in light of the additional effects of TM treatment of whole cells.
Selection of tumor cell variants with wheat germ agglutinin (WGA) has been used to produce sublines with different metastasizing, tumorigenic, and immunogenic properties. Some WGA-resistant variants are less tumorigenic \textit{in vivo} and are better stimulators and target cells in an \textit{in vitro} cytotoxicity assay. Changes in the membrane interactions of the H-2 antigens with cytoskeletal components are supposed to account for differences between variants, but the role of WGA in selecting these membrane variants is unclear (Dennis et al., 1981a; Dennis et al., 1981b). In another system, WGA selected a poorly metastasizing melanoma line whose cell-surface glycopeptides have less sialic acid and more fucose (Finne et al., 1980). A correlation between the biological properties of the variants and the structure of the carbohydrates on the cell-surface glycoproteins is evident in the above work, but the basis of the correlation is still unknown, and the evidence still somewhat indirect.

One further indirect line of evidence linking carbohydrate moieties to immune responses is the neuraminidase treatment of tumor cells to cleave the terminal, negatively charged sialic acid residues. The numerous investigations conducted using this enzyme have yielded apparently conflicting results. For example, neuraminidase-treated P815 target cells show no difference from untreated controls in susceptibility to CTL cytolysis.
(Weiss & Cudney, 1971). On the other hand, leukemia L 1210 cells treated with neuraminidase were 100 to 1000 times as immunogenic as radiation or formaldehyde treated cells (Holland & Bekesi, 1976). Weiss has suggested that the conflicting results seen in the various systems may be due to the distance between the sialic acid rich zones and the antigenic regions on the cell surface (Weiss, 1973).

Numerous studies implicate sialic acid (SA) with immunological responses. The processing of cell surface glycoproteins is very similar in both normal and transformed cells, but the transformed phenotype is associated with an increased capacity to produce more highly sialylated glycopeptides (Buck et al., 1974; Aoi & Yokota, 1978; Glick, 1979; Yogeeswaran et al., 1979; Ivatt et al., 1980).

Several malignant cell lines also have an increased SA content that correlates to a decrease in immunogenicity and an increase in tumorigenicity and/or metastases (Bryant et al., 1974; Warren et al., 1975; Kozlowska et al., 1976; Sirsat & Palekar, 1977; Whyte & Loke, 1978; Yogeeswaran et al., 1978; Manabe et al., 1979; Irimura et al., 1981; Portoukalian & Dore, 1981; Sherblom & Carraway, 1981; Vilarem et al., 1981). Interestingly, Gilmer et al. (1982) have reported that a CTL-susceptible EL4 ascites TC contains 2.2-fold less neuraminidase-releasable SA than a less CTL-susceptible EL4 tissue culture TC. When both the cell lines were treated with neuraminidase, conjugate formation
and lysis by allogeneic CTL were increased for both, suggesting some nonspecific role for the SA, possibly by general electrostatic shielding.

Evidence for antigen masking by large endogenous ectoglycoproteins containing SA has been presented for two ascites tumor systems: the strain A mouse mammary adenocarcinoma TA3 and a BALB/c mouse IgA-synthesizing plasmacytoma cell 58-8 (reviewed in Coldington, 1978).

Cowing and Chapdelaine (1983) have reported that treatment of allogeneic B cells with neuraminidase renders them mitogenic for T cells while normal resting B cells are not. T cells may therefore need to recognize the nonpolymorphic asialocarbohydrate moiety on the Ia molecules in order to be stimulated. A recent study using swainsonine, an inhibitor of oligosaccharide processing from the high mannose to the complex forms, supports this idea (Powell et al., 1983).

These studies on SA are particularly interesting because of the great variety of biological phenomena that can be ascribed to the sialic acids (reviewed in Reutter et al., 1982; Schauer, 1982). The sialic acid residue is relatively large, hydrophilic, and strongly acidic, a consequence of which is a hydration shell that enlarges the residue further. The cell surface forms of H-2k\textsuperscript{b} and H-2b\textsuperscript{b} do contain SA as terminal saccharides (Gilmer, 1982). Therefore, it is reasonable to postulate that the terminal
SA may modulate the recognition and/or function of the H-2 antigens. This modulation could be the direct result of the negative charge, or due to conformational alterations of the polypeptide to which it is attached caused by the negative charge. Electrostatic or steric masking of the antigenic regions could also occur, either directly, i.e., masking determinants on the same molecule, or indirectly, masking determinants on a different molecule.

In order to test the theory that sialic acid does influence the direct recognition of H-2 antigens, an asialo-H-2 antigen was isolated from a WGA-resistant variant of EL4 produced in this laboratory. The biological activity of the asialo-H-2K\textsuperscript{b} was compared to that of the sialylated H-2K\textsuperscript{b} using reconstituted lipid vesicles of defined composition. Conjugate formation between allogeneic CTL and TC was inhibited in an assay refined to minimize several problems previously encountered in such inhibition studies. The results reported here uniquely demonstrate in a direct assay that the terminal sialic acid residues of H-2K\textsuperscript{b} antigens do not influence the recognition of the antigen by allogeneic CTL.
MATERIALS AND METHODS

Mice. BALB/c female mice and C57BL/6J mice were obtained from either Charles River Laboratory, Wilmington, MA or our own breeding colony. Male C57BL/6J mice were used only to pass EL4 cells between experiments; female C57BL/6J mice were always used for growing cells for experimental purposes.

Cell lines. EL4 (H-2^b), a lymphocytic leukemia murine line derived from C57BL/6J, and the BW5147.3 (H-2^k) lymphoma line from a spontaneous AKR/J tumor were obtained from the Salk Institute, LaJolla, CA. The EL4 cells were maintained either in ascites form in the syngeneic C57BL/6J by passage of 5 x 10^6 cells intraperitoneally every 5-7 days or in tissue culture (t.c.) passed every 2-3 days by harvesting from growth at 37°C in a 5% carbon dioxide, 95% air incubator in Dulbecco's modified Eagle's medium (DMEM; Gibco No. 40-1600, Grand Island, NY) containing 10% heat-inactivated horse serum (HS; Gibco No. 230-6050) and 1% penicillin-streptomycin (PS; Eli Lilly Nos. 526 & 431, respectively, Indianapolis, IN). BW5147.3 line was grown identically to EL4 t.c. except when large cultures were needed; then the cells were grown in 500 ml and 1 liter spinner flasks. Cell viability typically was >98% for EL4 ascites and >90% for BW5147.3 and EL4 t.c.
The monoclonal antibody (mcAb) hybridomas M1/42.3.9.8 (M1/42; rat anti-mouse H-2, and, in our system, anti-H-2^k), 11-4.1 (anti-H-2^K^k), and B8-24-3 (anti-H-2^K^b) are available from the Salk Institute or the American Type Culture Collection, Rockville, MD. The hybridoma M1/42 cells were kept at high viability by growth in DMEM containing 5% heat-inactivated fetal calf serum (FCS; Gibco No. 230-6140) and 1% PS. 11-4.1 cells were maintained in DMEM containing 10% HS and 1% PS. B8-24-3 cells were maintained in DMEM containing 10% FCS and 1% PS.

Neuraminidase treatment and sialic acid assay. The procedure of Cödington et al. (1976) was used. Briefly, cells at a final concentration of (2-3) x 10^7 cells/ml were incubated for 80 min at 37° C (with occasional resuspension) in neuraminidase (V.cholerae; Calbiochem-Behring, La Jolla, CA) at a final concentration of 90-100 units/ml in a total volume of 1.40 ml. A unit of enzyme is defined by Mohr and Schramm (1960).

The sialic acid released in the neuraminidase treatment was determined by the thiobarbituric acid method of Warren (1959) using N-acetylneuraminic acid (Aldrich, Milwaukee, WI) as the standard. However, the procedure was modified by an additional centrifugation of the post-neuraminidase cell supernatant at 20,000 x g for 30 min to remove membrane vesicles before analyzing for the amount of sialic acid released.
WGA-selection of EL4 cells. A sterile stock solution of wheat germ agglutinin (WGA; 1.25 mg/ml from Bethesda Research Laboratories, Bethesda, MD) was serially diluted in sterile phosphate buffered saline (PBS; Gibco No. 450-1300). To each petri dish (60 x 20 mm) was added 4 ml of growth medium containing $2 \times 10^4$ EL4 t.c. cells, 200 µl of WGA dilution, and 0.8 ml agarose (type 1, low EEO, Sigma Chemical Co., St. Louis, MO) resulting in a final 0.3% agarose concentration. The petri dishes were cultured at $37^\circ C$ in a 5% carbon dioxide, 95% air incubator. Eight days later, surviving colonies were removed, grown in microtiter wells and expanded in culture. A total of 29 surviving colonies were isolated. A single colony surviving in the plate containing the highest concentration of WGA that allowed cell survival (12.5 µg/ml) was called WD1. WD1 grew in tissue culture with approximately the same doubling time as the parent EL4 t.c. line. The WD1 line was maintained in the presence of WGA (12.5 µg/ml) by weekly addition of WGA. When cells were needed for an experiment, the cells were washed twice in PBS at room temperature and then cultured for 2 to 9 days in the absence of WGA. However, the WD1 used for the isolation of H-2 antigens had been cultured about 2-4 months without WGA.

Membrane isolations, enzyme treatments, SDS-PAGE and two-dimensional gel electrophoresis. The methods used for the $^{35}$S-methionine labeling of cells, membrane isolations,
enzyme treatments, and sodium dodecylsulfate polyacrylamide
gel electrophoresis (SDS-PAGE) were identical to those
already described (Gilmer, 1982). Two-dimensional gel
electrophoresis was done by the modified method of O'Farrell
(1975), as given in Gilmer (1982).

Agglutination of WD1 and EL4 by various lectins. The
lectins were purified by standard procedures (Agrawal &
Goldstein, 1972; Sage & Green, 1972; Allen & Neuberger,
1975; Roche et al., 1975). All lectins were dialyzed at
approximately 0.60 mg/ml versus PBS except Limulus
polyphemus agglutinin (LPA) which was dialyzed at 0.18
mg/ml. Then 100 ul of each lectin (or a serial dilution of
it in PBS) was added to 1 x 10^6 EL4 t.c. or WD1 t.c. cells
in a total volume of 1 ml PBS. The cells were incubated for
10 min at 37° C and then centrifuged at 300 x g for 10 min
at 4° C. The degree of agglutination was determined
qualitatively by determining the size of the aggregate upon
attempting to resuspend the cells. The experiment was done
twice with very similar results.

M1/42 mcAb purification and coupling. When antibody was
needed, the cells were washed free of the serum and then
passed into the following serum-free medium (SFM) for 3-4
days. The SFM was DMEM containing 20 μg/ml human
transferrin (Calbiochem-Behring), 10 μg/ml bovine insulin
(Sigma), 20 μM ethanolamine (Sigma), 0.6 mg/ml L-glutamine
(Sigma), 17.3 ng/ml Na selenite (Aldrich), 5 mM lithium
chloride (Baker, Phillipsburg, NJ), 1% nonessential amino acids (Gibco # 320-1140), and 1% PS. These ingredients were obtained from SFM chosen for other cell lines (Iscove & Melchers, 1978; Chang et al., 1980; Imagawa et al., 1982; Murakami et al., 1982) and were tested in various combinations and concentrations with the M1/42 cells to arrive at the medium described above. Although cell viability dropped to 0-5% during the 3-4 days in the SFM, appreciable quantities of active mcAb were secreted into the medium.

A competitive ELISA assay using normal rat serum (a generous gift of Dr. Marc Freeman) and horseradish peroxidase-linked rabbit anti-rat IgG (H + L) (HRP-anti-rat IgG; Bionetics, Kensington, MD) was used to quantitate the relative amount of mcAb secreted into the SFM tested. The wells of a 96-well, flat bottom, polystyrene tissue culture plate were coated with normal rat serum diluted 1/500 in borate saline (BS; 0.10 M boric acid (Mallinckrodt), 0.025 M Na borate (Baker), 0.08 M NaCl, pH 8.4-8.5) overnight at 4°C. The coating solution was removed and could be saved for reuse. The wells were then blocked by filling with 1% (w/v) bovine serum albumin (Sigma) in BS (BS-BSA) and incubating for 1 hr at room temperature. The wells were then washed twice with 0.85% (w/v) NaCl and the wells aspirated dry. 50 to 100 µl of each sample was added to the well with 100 µl of the HRP-anti-rat IgG diluted 1/20 - 1/40 in BS-BSA, and
incubated 4 hr at room temperature or overnight at 4º C. The wells were washed 4–5 times with 0.85% NaCl and 200 µl of the substrate solution was added to each well. The following separate solutions were combined on the day of the assay to make the substrate solution: 15.35 ml 0.10 M citric acid (Fisher Scientific Co., Fair Lawn, NJ), 9.65 ml 0.20 M Na phosphate dibasic, 25 ml 0.2% (w/v) 2, 2'-azino-di-(3-ethyl-benz-thiazaline sulfonic acid) (ABTS; Sigma), and 5 µl 30% hydrogen peroxide (Mallinckrodt). The final pH should be 4.0. Following incubation at room temperature for at least 30 min, 100 µl 1.92 mg/ml NaF (Baker) was added to stop the reaction, and the absorbance at 414 nm was measured.

The actual total yields of isolated M1/42 varied somewhat depending on the initial viability of the cells when transferred to the SFM and on the total cell number transferred. For example, in one preparation, 5 x 10⁷ cells in 300 ml SFM at an initial viability of 90% yielded about 6.0 mg M1/42 mcAb. In another preparation, 1 x 10⁸ cells in 300 ml SFM at an initial viability of 67% yielded about 4.6 mg M1/42. Ideally, the highest cell number and initial viability should produce the most mcAb. The M1/42 cell-free supernatant was concentrated 100-fold with either an Amicon XM50 or PM10 filter, and the antibody was purified by recrystallization at room temperature three times from 50% ammonium sulfate (Baker).
An ELISA assay to determine antibody activity was adapted (Rook & Cameron, 1981) using EL4 t.c. cells in DMEM-10% HS-1% PS (DMEM-HS-PS) and HRP-anti-rat IgG to measure binding of the M1/42. EL4 t.c. were washed once in PBS, resuspended in DMEM-HS-PS, and adjusted to 1 x 10^7 cells/ml. To 12 x 75 mm borosilicate disposable culture tubes were added 0.5 ml DMEM-HS-PS, 0.15 ml cells, and 0.050 ml M1/42 mcAb at to 40 μg/ml in DMEM-HS-PS. Following a 30 min incubation at room temperature, the cells were spun down at 300 x g at room temperature for 10 min and washed once in DMEM-HS-PS. The cells were then resuspended in 0.7 ml DMEM-HS-PS and 100 μl of a 1/20 dilution of HRP-anti-rat IgG in BS-BSA was added. Following another incubation at room temperature for 30 min, the cells were spun down as above and washed twice in room temperature PBS. To the cells was added the same substrate solution, as described above. After one hr at room temperature, 50 μl 1.92 mg/ml NaF was added to stop the reaction, the cells were pelleted as above, and the absorbance of the supernatant at 414 nm was measured.

The M1/42 was quantitated by the Folin method (Lowry et al., 1951) using bovine serum albumin (Sigma) as the standard (using Abs at 280 nm of 0.667 for 1 mg/ml in a 1 cm pathlength cell (Lever, 1972)).

The M1/42 mcAb was coupled at 50 mg CNBr (Sigma)/1.0-2.0 mg antibody/ml Sepharose 4B (Sigma)
(Cuatrecasas, 1970). Lower levels of CNBr activation (25 mg CNBr/ml Sepharose 4B) yielded an affinity column that required harsher conditions to elute the H-2 antigens.

Purification of 11-4.1 mAb. The 11-4.1 mAb was purified using protein A affinity chromatography (protein A-Sepharose CL-4B, Pharmacia Fine Chemicals, Piscataway, NJ), as described previously (Herrmann & Mescher, 1979). Briefly, 11-4.1 cells grown in 500 ml spinner flasks were centrifuged at 300 x g for 10 min and the supernatant was applied to 1 x 1 cm protein A-Sepharose 4B column that had been pre-equilibrated with PBS, pH 7.5 containing 0.02% Na azide. The column was washed with the same buffer, and the antibody eluted with 50 mM glycine, pH 4.0. Fractions were collected into tubes containing several drops of 0.10 M Na phosphate, pH 7.0. Fractions containing antibody and the final antibody concentration were determined using an absorbance at 280 nm of 1.5 for 1 mg/ml (1 cm pathlength cell). Typical yields varied, probably as a function of cell viability which was not monitored. Three preparations of roughly the same volume of medium (600-650 ml) yielded 4.85 mg, 2.46 mg, and 1.60 mg. The 11-4.1 was coupled to Sepharose 4B using the same procedure as the M1/42 mAb.

Purification of B8-24-3 mAb. The B8-24-3 mAb was purified using protein A affinity chromatography, as previously described (Flores & Gilmer, 1984). Typically, one BALB/c mouse injected with 5 x 10^6 cells at 54% viability was
tapped three times yielding 1.3-1.5 mg mcAb each tapping. Antibody concentration was determined using an absorbance at 280 nm of 1.5 for 1 mg/ml (1 cm pathlength cell). The B8-24-3 was coupled to Sepharose 4B using the same procedure as the M1/42 mcAb.

H$_2^k$ purification. The H$_2^k$ antigen was obtained from BW1547.3 cells using an M1/42 mcAb-Sepharose 4B affinity column (0.9 x 2.0 cm) using the purification procedure of Stallcup et al. (1981), with some minor modifications. The detergent was Nonidet P40 (NP-40; Particle Data Laboratories, Elmhurst, IL) instead of Triton X-100, and a low salt wash (0.5% NP-40, 0.15 M NaCl (Baker), 20 mM Tris-HCl (Sigma), pH 8.0) was included before sample elution. The sample was eluted using 0.5% Na deoxycholate (Sigma), 0.65 M NaCl, 20 mM Tris-HCl, pH 8.0, as described (Stallcup et al., 1981; Mescher et al., 1983). The eluted antigen was diluted 2-fold with 20 mM Tris-HCl, pH 8.0 to prevent the buffer from gelling and immediately dialyzed versus 0.3% Na deoxycholate, 0.10 M NaCl, 20 mM Tris-HCl, pH 8.0, and concentrated 5-fold to 200-500 µl by vacuum dialysis. The H$_2^k$ was quantitated by the Folin method using bovine serum albumin as the standard, as described above.

A sample was radioiodinated to a specific activity of about 3.0 x 10$^3$ cpm/µg protein using the solid state lactoperoxidase (Lactoperoxidase from P-L Biochemicals,
Milwaukee, WI) method (David & Reisfeld, 1974). Free $^{125}$I-iodide was removed from the radiolabeled H-2 antigens by gel filtration on a Sephadex G-25 medium (Pharmacia Fine Chemicals) column (1.6 x 12 cm) at room temperature washed with 0.3% Na deoxycholate, 0.1 M NaCl, 20 mM Tris-HCl, pH 8.0. Prior to use in the liposome formulations, the $^{125}$I-antigens were concentrated in a Centricon 10 microconcentrator unit (Amicon, Danvers, MA) at 4°C, restored to the original volume and the radioactivity quantitated. This was repeated, typically three to four times, until the cpm in the retained protein was constant. Typically, (0.7-1.0) x $10^9$ cells were solubilized and purified yielding about 18 μg/10$^9$ cells.

Purification of H-2K$^b$ from EL4 and WD1. The H-2K$^b$ antigens were isolated from the EL4 and WD1 cell lines using the same solubilization procedure as described above by Stallcup et al. (1981). An 11-4.1 mcAb affinity column was used as an additional pre-column besides the standard Sepharose 4B pre-column. A BS-24-3 mcAb-Sepharose 4B affinity column (0.9 x 2.0 cm) was used to isolate the antigen, unless otherwise indicated, with a different set of washing and eluting buffers adapted from Albert et al. (1983). (H-2K$^b$ was occasionally purified using the M1/42 mcAb affinity column and a protocol identical to that for H-2K$^k$, as described above. The M1/42 mcAb recognizes only H-2K$^b$ and not H-2D$^b$ (Springer, 1980)). After loading the affinity
column with the clarified cell extract, the column was washed with 50-100 ml 0.25% NP-40, 20 mM Tris-HCl, pH 8.0 and then with 50-100 ml 0.25% NP-40, 0.15 M NaCl, 1 mM disodium (ethylenedinitrilo) tetraacetate (EDTA; Baker), 20 mM Tris-HCl, pH 7.4. The column was sequentially washed with 5 ml 1 M Tris-HCl, pH 7.8; 5 ml 0.3% Na deoxycholate, 0.10 M NaCl, 20 mM Tris-HCl, pH 8.0; 5 ml 1 M Tris-HCl, pH 7.8; and 5 ml 30 mM octyl glucoside (Calbiochem-Behring), 0.10 M NaCl, 2 M ammonium thiocyanate (Baker), 50 mM Tris-HCl, pH 7.4. The eluted antigen was immediately applied to a 1.6 x 12 cm Sephadex G-25 medium (Pharmacia Fine Chemicals) column at room temperature to remove the ammonium thiocyanate and washed with 0.3% Na deoxycholate, 0.10 M NaCl, 20 mM Tris-HCl, pH 8.0. Thereafter, the antigens were treated identically to the H-2^k^ antigens, as described previously, except the dilution step to prevent gelling was not necessary. Typically, (1-5) x 10^9 cells were solubilized yielding 12-14 μg H-2K^b^/10^9^ cells. The H-2 antigens were ^125^I-iodinated to a specific activity of about 2.0 x 10^4 cpm/μg protein and separated from ^125^I-iodide, as described above.

Isolation of cytoskeletal proteins. The cytoskeletal proteins (CP) were isolated from BW5147.3 (H-2^k^) lymphoma cells, as previously described (Mescher et al., 1981; Herrmann & Mescher, 1981a), except that dounce homogenizing of the pellets was introduced to insure complete
solubilization and washing of the pellet. Briefly, the plasma membrane fraction of (3-4) x 10^9 cells was dounce homogenized 20 times at 4° C in 10 mM Na phosphate (Mallinckrodt, Paris, KY), pH 7.0 buffer containing Earle's salts (PBES) and 0.2 mM phenylmethylsulfonylfluoride (PMSF; Sigma) (PBES-PMSF). The membranes were centrifuged at 87,000 x g for 45 min at 4° C. Pellets were resuspended by dounce homogenizing, as above, in 1 ml PBES-PMSF. The protein content was determined, as above, by a Folin assay using bovine serum albumin as the protein standard. The membranes were then solubilized by diluting PBES-PMSF containing 10% NP-40 into the sample in PBES-PMSF to a final protein concentration of 0.5 mg/ml and a final detergent concentration of 0.5% (w/v) and dounce homogenizing, as above. After incubation on ice for 15 min, the sample was centrifuged at 100,000 x g for 45 min at 4° C. Pellets were resuspended and dounce homogenized, as above, in PBES-PMSF and centrifuged at 100,000 x g for 45 min at 4° C. The washed, detergent-insoluble pellet, approximately 25% of the initial membrane protein, was resuspended by vortexing in 0.3% Na deoxycholate, 0.10 M NaCl, 20 mM Tris-HCl, pH 8.0 and frozen at -20° C. The CP preparations were checked for protease activity by incubating an aliquot 24-36 hours at 4° C and comparing that aliquot to a similar one frozen at -20° C for the same time period by one dimensional SDS-PAGE. The four major protein bands exhibited mobilities corresponding
Lipids and liposome formation. The lipid composition for the reconstitution was identical to that of Hollander et al. (1979). The stock solution contained 10 mg dipalmitoylphosphatidylcholine monohydrate (DPPC), 5 mg dimyristoylphosphatidylcholine monohydrate (DMPC), 3 mg cholesterol in 3 ml chloroform (Mallinckrodt; spectrophotometric grade) stored under nitrogen at -70°C. The cholesterol (Sigma) was purified using redistilled dichloromethane (Mallinckrodt) as the solvent on a Sephadex LH-20 (Pharmacia Fine Chemicals) column (Van Lier & Smith, 1969). It was stored under nitrogen at -70°C to prevent oxidation which causes immunosuppression in another immunological system (Humphries & McConnell, 1979). The phospholipids (Supelco, Bellefonte, PA) and purified cholesterol were shown to be pure by thin layer chromatography on 250 micron silica G60 (E. Merck Reagents, MC/B Manufacturing Chemists, Inc., Cincinnati, OH) plates using chloroform:methanol:water (65:25:4) and benzene:ethyl acetate (3:2) as the solvents, respectively. The position of the cholesterol was visualized by spraying with a 1:1 (v/v) solution of concentrated sulfuric acid (Mallinckrodt) and 100% ethanol (Florida Distillers Company, Lake Alfred, FL) and charring for 20 min at 105°C. A second plate was tested for the presence of peroxides by the ammonium thiocyanate-ferrous sulfate spray (Waldi, 1965).
The phospholipids were developed using Dragendorff's reagent (Waldi, 1965). Observed Rf values were 0.49 for DPPC, 0.52 for DMPC, and 0.56 for cholesterol. The purified cholesterol was quantitated using the Lieberman-Burchard reaction (Stadtman, 1957).

Typically, the liposomes were prepared by first drying a total of 60 µg of lipid as a film three times under nitrogen from chloroform. The film was dissolved in a solution containing 25 µg CP plus 5 µg H-2Kb (or 5 µg H-2k) doped with a small amount of 125I-H-2 in 0.250 ml 0.2-0.5% Na deoxycholate, 0.15 M NaCl, 20 mM Tris-HCl, pH 8.0, so that the initial total lipid:total protein ratio was 2:1 (wt:wt). The resulting solution was dialyzed for 36-48 hours using three 0.5 liter changes of PBS at 4°C. The second PBS buffer also contained 1% PS. The final CP/H-2 ratio in the recovered liposomes, as determined by total protein determinations (Folin assay) and H-2 content (recovered radioactivity), varied from (2-6)/1. The final lipid to protein ratio was not determined.

Sucrose density gradient centrifugation. Liposomes were prepared, as described above, except that the lipid preparation also contained 420 nCi tracer 14C-cholesterol (New England Nuclear, Boston, MA). To 100 µl of the liposome preparations was added either 100 µl PBS-0.02% Na azide or 100 µl of a 1 mg/ml Pronase E (Streptomyces griseus, type XIV, Sigma) dissolved in PBS-0.02% Na azide,
and incubated for 2 hr at room temperature. Solid sucrose (Baker) was added to bring the sucrose concentration to 55% (w/w), and a 0%-50% (w/w) sucrose gradient in PBS-0.02% Na azide was layered on top. The gradients were centrifuged for 18 hr at 180,000 x g at 4° C. Twenty drop fractions were collected from the top of the centrifuge tubes and assayed for 14C-cholesterol by scintillation counting and for 125 I-H-2 by gamma counting. Sucrose concentrations were determined with a refractometer.

Inhibition of conjugate formation. The same basic procedure, as described previously (Gilmer et al., 1978), was used except the T cells and targets cells were resuspended in Ca2+-free PBS containing 5 mM ethyleneglycol-bis-(beta-aminoethyl ether) N,N'-tetraacetate (EGTA; Sigma) and 5% FCS after isolation and fluorescein diacetate loading, respectively, instead of PBS-5% FCS. In addition, the T cells were preincubated with the liposomes as described below, and the conjugates were formed by centrifugation for 15 min at 1325 x g using a swinging bucket rotor at room temperature (27-29° C). The T cells were primed allogeneically (BALB/c anti-EL4; H-2d anti-H-2b) and purified from the BALB/c, peritoneally derived cells on a nylon wool column (Gilmer et al., 1982). The cytolytic activity was shown to be T cell-mediated by abrogation of 51Cr release upon pretreatment of the T cells with anti-Thy-1.2 plus complement (Gilmer et al., 1982). Each
sample was prepared in 35 µl, and the components were combined in the following manner: vesicles containing H-2 antigens plus CP were mixed with 3.16 x 10^4 T cells and incubated at 37°C in a 5% carbon dioxide, 95% air incubator for one hour. After ten minutes at room temperature, 1.58 x 10^4 fluorescein diacetate-loaded target cells were added to the mixture resulting in an effector/target ratio of 2/1. The cells were spun for 15 minutes at room temperature, as described above, to form conjugates. Only 7 µl of this sample was needed for microscopic examination. The % conjugate formation was determined as the % T cells which were bound to fluorescent target cells. A total of 200 to 400 T cells were counted per sample. Repeat counts of identical samples were within 3% of each other.
RESULTS

Carbohydrate forms of the antigens present on the cell surface of WD1 variant. A WGA-resistant variant of the murine EL4 lymphocytic leukemia cell line, designated WD1, was selected for low levels of cell-surface sialic acid. To assay the nature of the carbohydrate differences on the cell surfaces of EL4 and WD1, the agglutinability by lectins was compared for EL4 and WD1. WGA agglutinated EL4 more strongly than WD1 at all concentrations tested (Table I). Similarly, LPA agglutinated EL4 more strongly than WD1 at all but the lowest lectin concentration. This was expected since WD1 was selected from EL4 by the toxic effects of WGA, and both WGA and LPA can recognize sialic acid (Table II).

Total cell-surface sialic acid, as measured by the Warren assay (as modified in Gilmer et al., 1982), was 26% lower in WD1 (0.46 ± 0.03 µmol/10⁹ cells) than in EL4 t.c. (0.62 ± 0.06 µmol/10⁹ cells).

In contrast to WGA and LPA, SBA agglutinated WD1 significantly better than EL4, and LcR failed to discriminate between the two cell lines. Concanavalin A (Con A) agglutinated EL4 significantly better than WD1 at 15 µg/ml and lower.

Two-dimensional gel analysis had previously shown the H-2 antigens on the cell surface of the WD1 were not
<table>
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<th>Lectin</th>
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\(^a\) Lectins: Con A, Concanavalin A; LcH, lentil lectin; LPA, \textit{Limulus polyphemus} agglutinin; SBA, soybean agglutinin; WGA, wheat germ agglutinin.

\(^b\) Scoring of agglutination: +4, associated mass of cells; +3, large cell aggregates; +2, small cell aggregates; +1, smaller cell aggregates; -, no aggregates (same as untreated control cells).

\(^c\) ND, not determined.
<table>
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<th>Lectin</th>
<th>Source</th>
<th>Sugar specificity</th>
<th>References</th>
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<td>Concanavalin A</td>
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<td>N-acetyl-neuraminic acid</td>
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sialyated (Figure 2; Gilmer et al., 1984). Those observations were extended to the mcAb purified H-2K<sup>b</sup> antigen from EL4 with the same results (Figure 3; cf. details in Results below).

Selection of SFM for M1/42 mcAb production. The M1/42 (rat anti-mouse H-2) mcAb is an IgG2a antibody containing specific H and L and myeloma K chains. Since it is not bound by protein A (Ey et al., 1978), a serum-free medium to facilitate purification of the mcAb from t.c. cells was formulated by testing a number of different ingredients in different combinations (Table III). Because the initial viability was not always the same, a normalized viability score (NVS) was calculated on a scale of 0 to 10, with a score of 0 indicating 0% viability at the end of the 3 days and a score of 10 indicating no drop in viability. Similarly, the ELISA absorbance values were normalized in such a way that the higher the number, the more mcAb present in the SFM being tested. (The ELISA was a competitive assay which gave lower values for wells with mcAb than for wells without mcAb.) In choosing the SFM to be used, both the NVS and the amount of mcAb secreted were optimized simultaneously, as best as possible. SFM number 39 containing 20 μg/ml human transferrin, 10 μg/ml bovine insulin, 20 μM ethanolamine, 0.6 mg/ml L-glutamine, 17.3 ng/ml Na selenite, 5 mM LiCl, 1% nonessential amino acids,
Figure 2. Autoradiograms of two-dimensional gels (10% acrylamide) of $^{35}$S-methionine-labeled plasma membrane (PM) preparations. (A) PM of EL4 t.c., (B) PM of EL4 t.c. treated with neuraminidase and a protein phosphatase, (C) PM of WD1. The basic end of the isoelectric focusing gel is on the left, and the direction of the SDS electrophoresis is from top to bottom. The spot labeled A in each gel is actin and provides an internal marker of $M_r = 45,000$ and isoelectric point 6.2. The molecular weight range covered in these gels is from 75,000 (top) to 26,000 (bottom), and the isoelectric range is from 6.6 (on left) to 5.3 (on right). In gel (A) the positions of the four sialylated forms of H-2$^b$ are indicated by the tilted bracket and of the two nonsialylated H-2$^b$ species by the level bracket. The more basic of the two nonsialylated species is H-2D$^b$, and the more acidic is H-2K$^b$, as determined by immunoprecipitation (Gilmer, 1982). In gels (B) and (C) the same region as the level bracket of gel (A) is indicated similarly.
Figure 3. Autoradiogram of a two-dimensional polyacrylamide (10%) gel of H-2K<sup>b</sup> from EL4 (1 μg; 2550 cpm), purified on an M1/42 mcAb affinity column. The basic end of the isoelectric focusing gel used in the first dimension is on the left and the acid end is on the right. The direction of the SDS electrophoresis used in the second dimension is from top to bottom. The spot labeled A in each gel is actin and provides an internal marker of M<sub>r</sub> = 45,000 and isoelectric point 6.2. The arrow points to the isoelectric form of the H-2K<sup>b</sup> containing three sialic acid moieties per polypeptide chain.
TABLE III: Selection of Serum-free Medium\textsuperscript{a}

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\textsuperscript{a} Abbreviations: I, insulin, \( \mu g/ml \); T, transferrin, \( \mu g/ml \); Se, 2.5 mM Na selenite; E, 20 \( \mu M \) ethanolamine; G, 0.6 mg/ml L-glutamine; AA, 10 mM nonessential amino acids; Li, 5 mM LiCl; BSA, 400 \( \mu g/ml \) bovine serum albumin.
Normalized viability score = cell viability after 3 days in SFM times 10 divided by cell viability at day zero.

Normalized mAb secretion score = ELISA absorbance with no mAb present divided by ELISA absorbance with SFM being tested.
and 1% F8 appeared to be the best and was chosen for large scale mcAb production.

Purification of M1/42. Transferrin and insulin are the only two major proteins other than the mcAb in the SFM. Further purification of the SFM-mcAb was desired to reduce the amounts of these two proteins and to remove any protease activities associated with the high cell death in the SFM by the time of harvest. The results of the purification of M1/42 are shown in Figure 4, lanes 1-3. In the ammonium sulfate precipitation, the volume was restored to the starting volume after solubilizing the pellet. Then aliquots of equal volume were analyzed by SDS-PAGE. Therefore, a comparison of these lanes gives a good measure of the purification as well as the yield of mcAb.

Transferrin (Mr = 80,000) is the principal protein in the concentrated SFM, and it is almost totally removed by the ammonium sulfate precipitations. This particular preparation was concentrated by vacuum dialysis instead of by ultrafiltration, so the insulin was retained. Concentration by ultrafiltration removes most of the insulin. The final product using ultrafiltration was very pure and exhibited a heavy chain of Mr = 52,600 and a light chain of Mr = 25,100.

Purification of B8-24-3. The final purified B8-24-3 mcAb contained only three bands (Figure 4, lane 4): a
Figure 4. One-dimensional sodium dodecylsulfate polyacrylamide (10%) gel electrophoresis of M1/42 mcAb purification, purified B8-24-3 mcAb and 11-4.1 mcAb, and CP preparation.

A. Purification of M1/42 mcAb from SFM; the gel was stained with Coomassie brilliant blue: 100-fold concentrate of SFM after 4 days of cell growth (lane one); first and third recrystallized M1/42 mcAb preparations, respectively (lanes two and three). Equivalent volumes at each step were used for this gel (see text for details). Arrowheads indicate protein standards: bovine serum albumin (Mr = 68,000), ovalbumin (Mr = 45,000), α-chymotrypsinogen (Mr = 25,700), and ribonuclease A (Mr = 13,700).

B. B8-24-3 mcAb purified by protein A affinity chromatography; the gel was silver stained: 1.0 μg B8-24-3 mcAb (lane four). 11-4.1 mcAb purified by protein A affinity chromatography; the gel was silver stained: 1.0 μg 11-4.1 mcAb (lane 5). Arrowheads indicate protein standards: bovine serum albumin (Mr = 68,000), ovalbumin (Mr = 45,000), trypsinogen (Mr = 24,000), and lysozyme (Mr = 14,300). The two diffuse bands corresponding to Mr 60,000 and 66,000 were artifacts of the silver staining procedure and also appeared in lanes containing only the Laemmli (Laemmli, 1970) solution.

C. CP tested for proteolytic activity; the gel was silver stained: 2.5 μg CP incubated overnight at 4°C (lane
6); 2.5 μg CP incubated overnight at -20° C (lane 7). Arrowheads indicate protein standards as in Figure 4B. Arrows indicate major CP proteins at Mr = 69,000, 67,700, 45,600, and 34,200. Open arrows indicate the heavy and light chains of the putative H-2^k contaminants.
contaminant at Mr = 67,200, presumably an albumin, the mcAb heavy chain at Mr = 53,500, and the mcAb light chain at Mr = 25,100. The light chain exhibited negative silver staining on gels more heavily loaded than the one shown.

**Purification of 11-4.1.** The final purified 11-4.1 mcAb contained only two broad bands (Figure 4, lane 5): the mcAb heavy chain at Mr = 48,900-52,400, and the mcAb light chain at Mr = 24,300-27,600. These bands appear to be broader than normal previously seen with this antibody. The reason is unknown at this time.

**Purification of H-2 antigens.** Previous work (see discussion) has shown that a mcAb coupled to Sepharose 4B can be used for affinity purification of H-2 proteins. The silver-stained one-dimensional gels shown in Figure 5 demonstrate that both the heavy and light chains of H-2 are greatly enriched in the eluate and are free of any major contaminants. The heavy chain of H-2\(^{k}\) exhibited a Mr = 47,600 - 50,300, while that of H-2\(^{k}\) from EL4 a Mr = 47,000 - 48,800, and that of H-2\(^{k}\) from WD1 a Mr = 47,100 - 48,200. The light chain exhibited Mr = 12,100, 13,400, and 13,400 for each of the three preparations, respectively. Both H-2\(^{k}\) preparations contain a slight amount of contamination with a protein, most likely actin (Gilmer, 1982), at Mr = 45,000 and a protein at Mr = 14,500.

Two-dimensional gel analysis of an \(^{125}\)I-radiolabeled preparation of H-2\(^{k}\) from EL4 (purified on an M1/42 mcAb
Figure 5. One-dimensional sodium dodecylsulfate polyacrylamide (10%) gel electrophoresis of H-2 preparations: 1.2 μg purified H-2^k, purified on M1/42 affinity column (lane one); 1.3 μg purified H-2^k^ from WD1, purified on BS-24-3 mcAb affinity column (lane two); 1.4 μg purified H-2^k^ from EL4, purified on BS-24-3 mcAb affinity column (lane three). Arrowheads indicate positions of protein standards. The two larger protein standards were identical in all lanes: bovine serum albumin (Mr = 68,000), and ovalbumin (Mr = 45,000). The two smaller protein standards were α-chymotrypsinogen (Mr = 25,700), and ribonuclease A (Mr = 13,700) in lane one and trypsinogen (Mr = 24,000), and lysozyme (Mr = 14,300) in lanes two and three. The two diffuse bands corresponding to Mr 60,000 and 66,000 were artifacts of the silver staining procedure and also appeared in lanes containing only the Laemmli (Laemmli, 1970) solution.
column) showed that, in comparison with two-dimensional gels of previously reported H-2 b immunoprecipitates (Gilmer, 1982), the principal species were two different isoelectric forms corresponding to H-2K b containing complex oligosaccharides with two and three (see arrow on Figure 3) sialic acid moieties per polypeptide chain (Figure 3). There was no endoplasmic reticulum form of H-2K b apparent (Gilmer, 1982) in the preparation, even upon prolonged exposure of the gel to an autoradiogram. The only detectable contaminant was a small amount of actin (see species labeled A on gel). It is unlikely that the different carbohydrates on the H-2K b from EL4 and WD1 affect their binding to the mcAb used for their isolation (Shimada et al., 1970; Nathenson & Cullen, 1974; Ploegh et al., 1981).

**CP isolation.** Solubilization of the plasma membrane of BW5147.3 cells yielded a detergent-insoluble fraction containing four principal cytoskeletal proteins having apparent molecular weights of 69,000, 67,700, 45,600, and 34,200 (Figure 4, see arrows in lanes 6 and 7). Prior to the introduction of dounce homogenization to the preparation protocol, some problem with autoproteolysis of the CP was evidenced by the disappearance of the higher molecular weight bands and the increase in intensity and number of lower molecular weight bands on similar one-dimensional
Figure 6. Sucrose density gradient (0-55%) profiles of liposomes containing tracer $^{125}$I-iodinated H-2K$^b$ ($\triangle$) and tracer $^{14}$C-labeled cholesterol ($\bullet$). Liposomes containing H-2K$^b$ (2.5 µg) were prepared with the standard lipid composition either without or with CP (25 µg). They were pretreated with Pronase E or control buffer and subjected to sucrose density gradient centrifugation. Fractions were collected from the top of the tubes and assayed for $^{125}$I and $^{14}$C to determine the amount of $^{125}$I-H-2K$^b$ and $^{14}$C-cholesterol present, respectively. (A) Liposomes prepared from H-2K$^b$ and lipid. (B) Liposomes prepared from H-2K$^b$ and lipid, pretreated with Pronase E. (C) Liposomes prepared from H-2K$^b$, CP, and lipid. (D) Liposomes prepared from H-2K$^b$, CP, and lipid, pretreated with Pronase E. The inverted triangles ($\triangledown$) indicate locations of peaks in a control tube containing H-2K$^b$ alone and treated as in (A) or (C). Those peaks at fractions 5, 17, and 20 contained 273, 105, and 784 cpm, respectively. All cpm values have been corrected for background. The density (○) of each fraction (see A) was calculated from the refractive index. The tubes which contained vesicles are indicated by a bar in each plot.
gels. A comparison of lanes 6 and 7 in Figure 4 shows that the above modification appears to have solved that problem. The lanes in Figure 4 were overloaded for the silver staining technique so many minor bands were also apparent. Of these, two are bands possibly corresponding to H-2\textsuperscript{k} antigens at Mr = 49,800 and Mr = 12,500 (see open arrows in Figure 4). The degree of contamination by presumptive H-2 antigens varied from preparation to preparation and was also noted in the preparation of CP from EL4. For this reason, BW5147.3, an irrelevant target cell for the CTL, was chosen as the source of CP in all experiments reported here.

Characterization of liposomes. H-2K\textsuperscript{b}-containing liposomes formed in the presence or absence of CP were analyzed by centrifugation on a 0-55\% continuous sucrose density gradient (Figure 6). Liposomes containing only H-2K\textsuperscript{b} and lipid floated near the top of the sucrose gradient at a density of about 1.03-1.06 gm/cm\textsuperscript{3} (see bar in Figure 6A). Liposomes made from H-2K\textsuperscript{b}, lipid, and CP floated in an heterogeneous manner in a density range of 1.03-1.14 gm/cm\textsuperscript{3} (see bar in Figure 6C). Also note that fraction 3 in Figure 6A of the CP-free vesicles contained a significant amount of \textsuperscript{125}I-labeled H-2K\textsuperscript{b} but no \textsuperscript{14}C-labeled cholesterol. Only 32\% of the \textsuperscript{125}I-H-2K\textsuperscript{b} was incorporated into liposomes in the absence of CP. However, when CP were present, 59\% of the \textsuperscript{125}I label was in the cholesterol-containing vesicles.
Figure 7. Inhibition of T cell-target cell conjugate formation by lipid vesicles reconstituted either with purified H-2K\textsuperscript{b} plus CP (●) or with H-2\textsuperscript{k} plus CP (■). Conjugate formation was measured by the % T cells conjugated to fluorescent target cells. The % conjugate formation with the same T cells and non-specific BW\textsuperscript{5147.3} (H-2\textsuperscript{k}) targets in this experiment was 2%. The final calculated ratio of CP to H-2 was 4.4/1 for H-2\textsuperscript{k} and 4.6/1 for H-2K\textsuperscript{b}. The amount of H-2 antigen indicated (as measured by recovered \textsuperscript{125}I) is the amount added to the 7 µl volume that was analyzed under the epifluorescence microscope.
In order to determine the degree of vectorial orientation of the H-2K<sup>b</sup> antigen toward the outside of the vesicles (and, therefore, available for interaction with CTL), the vesicle preparations were pretreated with Pronase E and then centrifuged in the sucrose gradient (Figure 6B and 6D). In a comparison of Figures 6A with 6B and of 6C with 6D, it appears that CP augmented the susceptibility of the vesicularized H-2K<sup>b</sup> to proteolytic digestion. Approximately 40% of the antigen in vesicles containing CP were cleaved while none of the antigen in vesicles without CP was cleaved.

Inhibition of conjugate formation: the method. Similarly prepared lipid vesicles containing H-2K<sup>b</sup> (or H-2<sup>k</sup>) and CP were used in the inhibition of conjugate formation experiments. 5 ng of H-2K<sup>b</sup> plus 23 ng CP (or 28 ng total protein) yielded 50% of the maximum observed inhibition of conjugate formation (Figure 7). Reproducible experiments were attained as long as freshly prepared vesicles were utilized in the assay. Similar results were obtained with three different preparations each of CP, H-2<sup>k</sup>, and H-2K<sup>b</sup> in a total of six experiments.

Inhibition of conjugate formation was observed with the control vesicles reconstituted with H-2<sup>k</sup> plus CP, but only at approximately 5- to 7-fold higher concentrations of H-2<sup>k</sup> than observed with H-2K<sup>b</sup> (Figure 7). This type of cross
Figure 8. Inhibition of T cell-target cell conjugate formation by lipid vesicles reconstituted either with purified H-2\textsuperscript{k} plus CP (■), with purified H-2K\textsuperscript{b} from EL4 plus CP (●), or with purified H-2K\textsuperscript{b} from WD1 plus CP (△). The % conjugate formation with the same T cells and non-specific BW5147.3 (H-2\textsuperscript{k}) target cells in this experiment was 0.5%. The final calculated ratio of CP to H-2 was 1.8/1 for H-2\textsuperscript{k}, 2.1/1 for H-2K\textsuperscript{b} from EL4, and 1.6/1 for H-2K\textsuperscript{b} from WD1.
reactivity was also observed in inhibition of conjugate formation between fluorescently labeled EL4 target cells and BALB/c anti-EL4 CTL by unlabeled P815 (H-2^d) or YAC (H-2^a) target cells (Schick & Berke, 1979).

Control target cells (BW5147.3) of a different H-2 background (H-2^k) were mixed with the same CTL that had been primed to recognize H-2^b targets to determine the degree of non-specific conjugate formation. Vesicles containing CP and H-2K^b only inhibit specific conjugate formation and had no effect on non-specific conjugate formation using the BW5147.3 target cells (data not shown). Vesicles containing CP and H-2^k also had no effect on non-specific conjugate formation (data not shown).

The specific inhibition of conjugate formation by vesicles containing H-2K^b and CP never approached the limiting value, i.e., the % conjugate formation observed with non-specific target cells.

Inhibition of conjugate formation: H-2K^b from EL4 vs. H-2K^b from WD1. The system described above was used to compare the recognition of H-2K^b antigens from the EL4 and WD1 cell lines. The only difference between the two liposome preparations should be the sialylation level of the H-2 antigens: H-2K^b from EL4 having 2-3 and H-2K^b from WD1 having no sialic acid residues per polypeptide chain. No significant difference in inhibition was noted between the two (Figure 8). About 4.0 ng of H-2K^b from EL4 was required
to achieve 50% inhibition, and only 4.4 ng of H-2K \textsuperscript{b} from WD1. Similar results were obtained in two experiments with two different WD1 preparations.
DISCUSSION

Characterization of cell-surface carbohydrates on EL4 and WD1. One approach to determining the role of carbohydrates in the structure and function of cell-surface glycoproteins is to select variant cell lines that differ in oligosaccharide processing. The most common selection method uses plant lectins (reviewed by Briles & Kornfeld, 1978; Stanley, 1980). The WD1 variant, characterized in detail elsewhere (Gilmer et al., 1984), was selected by the direct toxic effects of the lectin, WGA, on the EL4 lymphocytic leukemia t.c. cell line. For this work, the goal was to isolate a variant which expressed nonsialylated H-2b molecules on the cell surface in order to purify and reconstitute the H-2 antigens into liposomes. The biological activity of the H-2 antigens could then be compared as a function of carbohydrate structure only.

Initially, the carbohydrates on the cell surface of the selected WD1 variant and the EL4 parent were compared in a general way by the ability of various lectins to agglutinate the cells (Table I). The increased agglutination of EL4 cells by both WGA and LPA suggested that sialic acid and/or N-acetylglucosamine residues were more available for agglutination in EL4 than in WD1. The increased agglutination of EL4 by Con A suggested that internal
mannose and/or glucose residues were more available in EL4 than in WD1. In contrast, the increased agglutination of WD1 by soy bean agglutinin suggested that more galactose residues were available in WD1 than in EL4. LcR did not distinguish between the two cell types probably because its sugar specificity is more in keeping with it binding to the internal core sugars of N-asparagine oligosaccharides (Table II). These sugars on the cell surface are probably to a great extent inaccessible to the LcR, and the minor changes in the carbohydrate suggested here would not be adequate to alter the agglutination properties of the cells very much. The LcR results do argue for only minor changes in the overall surface carbohydrates. The agglutination results taken together suggest that various carbohydrates differ on the cell surfaces of WD1 and EL4.

The two-dimensional gel data (Figure 2; from Gilmer et al., 1984) demonstrated that the WD1 line does not deliver sialylated, $^{35}$S-methionine-labeled H-2^b proteins to the cell surface during the 90 min labeling period (Fig. 2C), whereas the parental EL4 line does (Fig. 2A). The ladder of isoelectric species primarily attributable to the presence of variable amounts of sialic acid per H-2^b polypeptide chain was totally absent in the WD1 line (cf. Fig. 2C with Fig. 2A). Instead, there was a protein (Fig. 2C) which had very similar isoelectric mobility to one of the two major species resulting from the action of neuraminidase and
protein phosphatase on the PM fraction of the parent EL4 line (Fig. 2B). A two-dimensional gel of WD1 cell surface proteins labeled by lactoperoxidase-catalyzed $^{125}$I-iodination showed no sialylated H-2$^b$ species (M. Sullivan and P.J. Gilmer, unpublished data).

Gilmer et al. (1984) have also shown a lack of reactivity of the plasma membrane forms of H-2$^b$ from WD1 with endoglycosidase H (endo H), suggesting that these carbohydrates were either complex, nonsialylated oligosaccharides, or endo H-insensitive high mannose forms (Gilmer, 1982; cf. Fig. 1). The former alternative is supported by the higher binding of SBA to WD1 than to EL4 (Table I), which indicates an increase in terminal galactose residues characteristic of complex, nonsialylated oligosaccharides. The decreased binding of Con A to WD1 also argues against endo H-insensitive high mannose forms on WD1, as do the results with LcH. It therefore appears that the goal of producing a source of complex, nonsialylated H-2$^b$ molecules has been attained.

Serum-free medium and M1/42 mcAb purification.

Traditionally, tissue culture media have required serum supplementation in order to support long-term culture of cells. However, serum is a complex mixture of components which may vary according to the age, sex, and species of its source. It is the major source of protein contamination when seeking to purify secreted monoclonal antibodies from
the spent medium. Sato and his co-workers have systematically investigated the hormone and growth factor requirements of a large number of cell lines. They completely defined serum-free media that promote growth comparable to serum-containing media and in which cells maintain their differentiated characteristics (Bottenstein et al., 1979; Barnes & Sato, 1980). Among the lines investigated are the mouse myeloma MPC11 (Murakami et al., 1981), rat neuroblastoma B104 (Bottenstein & Sato, 1979), and human colon carcinomas (Murakami & Masui, 1980).

Murakami et al. (1982) devised a serum-free medium supplemented with a few growth factors to grow mouse plasmacytomas and hybridoma cell lines of MPC11-BL and SP2/0-BL origins. They found that insulin (5 μg/ml), transferrin (35 μg/ml), ethanolamine (20 μM), and selenium (2.5 nM) are necessary supplements to support long-term cultivation of their cell lines. They did note that hybridoma cell lines made by the fusion of plasmacytoma NS-1 with either mouse or rat spleen cells could not grow sufficiently in their medium. The M1/42 hybridoma is a fusion of NS-1 to DA rat spleen cells, so additional components besides the four mentioned above were tested for M1/42.

Chang et al. (1980) succeeded in growing several established hybridoma cell lines in a serum-free medium supplemented with physiological concentrations of insulin (5
μg/ml) and transferrin (5 μg/ml). Their formulation also included L-glutamine (0.6 mg/ml) and 1% nonessential amino acids.

A serum-free medium for growth of normal and tumor mouse mammary epithelial cells in primary culture has been reported (Imagawa et al., 1982). It contains insulin (10 μg/ml), epidermal growth factor (EGF; 0.01 μg/ml), transferrin (10 μg/ml), BSA fraction V (5 mg/ml) and cholera toxin (0.01 μg/ml). The authors noted that 5-10 mM LiCl could replace EGF, but then insulin and albumin were definitely required.

Based on the above work, the following components were tested in various combinations to determine if a serum-free medium for long term culture of the M1/42 hybridoma could be developed: several concentrations of insulin and transferrin, selenium, ethanolamine, L-glutamine, nonessential amino acids, LiCl, and BSA (Table III). The combination which appeared best to optimize cell viability and mcAb production simultaneously (medium # 39) was one which included all the tested components except BSA. In agreement with Murakami et al. (1982), we could not achieve long term culture of the M1/42 hybridoma. However, adequate mcAb production for our purposes was achieved (about 4-6 mg per 0.5-1.0 x 10^8 cells in a typical preparation). Standard purification procedures were greatly simplified with the lower level of protein contamination, and greater
purification was achieved with less effort than from standard serum media.

**H-2 antigen purification.** With the availability of many mcAb hybridomas specific for the major histocompatibility (MHC) antigens, it has become possible to rapidly purify the antigens with good yields. Using affinity columns of the mcAb coupled to Sepharose, the MHC antigens can be readily purified from detergent-solubilized whole cells or cell membranes. H-2 (Herrmann & Mescher, 1979; Stallcup *et al.*, 1981) and HLA-A, -B, -C, and -DR antigens (Parham, 1979; Walker & Reisfeld, 1982) of a number of specificities as well as rat Ia (McMaster & Williams, 1979) and murine I-A<sup>k</sup> and I-E<sup>k</sup> (Turkewitz *et al.*, 1983) have been purified on a relatively large scale in this way.

Affinity columns of M1/42, a rat anti-mouse H-2 mcAb having no haplotype specificity (Stallcup *et al.*, 1981), have been used to purify relatively large quantities of biologically active H-2 antigens from the b, d, and k haplotypes. In this study, the H-2<sup>k</sup> antigens were purified for use as a control in the inhibition of conjugate formation experiments. Mild elution conditions (0.5% Na deoxycholate and 0.65 M NaCl, pH 8.0) result in yields of 70-90% of serologically and biologically active antigens (Stallcup *et al.*, 1981). Our yields (cf. Methods and Materials) and purity (Figure 5) appear comparable to those previously reported, though yields are to some extent a
function of the cell line used for an antigen source. Slightly higher yields may have been obtained for the $H^{-2k}$ antigens (18 μg/10^9 cells vs. 12-14 μg/10^9 cells for $H^{-2K^b}$) because the M1/42 mcAb reacts equally well with both $H^{-2K}$-end and $H^{-2D}$-end products of the $k$ haplotype, whereas it recognizes only the $H^{-2K}$-end product of the $b$ haplotype, as does the haplotype specific anti-$H^{-2K^b}$ mcAb (B8-24-3) used to isolate the $H^{-2K^b}$ antigens in this work.

No protocol has been published to date for the immunopurification of $H^{-2K^b}$ molecules from a B8-24-3 mcAb affinity column. When elution was performed using Na deoxycholate and NaCl, as used with the M1/42 mcAb affinity column, no $H^{-2K^b}$ could be recovered from the B8-24-3 immunoadsorbents (data not shown). Albert et al. (1983) have reported a procedure for a different anti-$H^{-2K^b}$ mcAb (mcAb 20-8-4) using harsher elution conditions which still yields serologically and biologically active $H^{-2}$ antigens. NP-40 lysates were first passed on an anti-$H^{-2K}$ affinity precolumn to remove components binding non-specifically to immunoglobulin, and then on their anti-$H^{-2K^b}$ affinity column. About 70% of the $H^{-2K^b}$ activity, as detected by a solid-phase RIA, could be eluted from their anti-$H^{-2K^b}$ immunoadsorbent in the presence of 2 M ammonium thiocyanate and 30 mM octyl glucoside (pH 7.4). Reconstitution of their purified $H^{-2K^b}$ molecules yielded liposomes which could inhibit cytolysis by antibody plus complement and could
stimulate in vivo primed T cells to generate cytotoxic T lymphocytes in vitro.

Using basically the same protocol for antigen elution, we were also able to obtain pure H-2Kb antigens from the B8-24-3 mcAb affinity column. In other studies, the number of H-2Kb molecules per ascites cell surface has been quantitated as 2.0 x 10^5 for EL4 and 1.5 x 10^5 for WD1 (Gilmer et al., 1984). Theoretically, one would predict that 20 pg of H-2Kb could be isolated from the cell surface of 10^9 EL4 cells and 15 pg from WD1. In fact, the yield of the isolated antigen was 12-14 pg/10^9 cells, or 60-70% of the theoretical maximum. This is in close agreement with the empirical results of 70% given by Albert et al. (1983). Stallcup et al. (1981) reported a yield of 25 pg/10^9 from EL4 cells using an M1/42 immunoadsorbent column.

Cytoskeletal proteins. The generation of a CTL response requires recognition of the cell surface antigens of the stimulator cells. Several studies (Fast & Fan, 1978; Lemonnier et al., 1978; Mescher et al., 1978; Hale, 1980) have demonstrated stimulation of an allogeneic CTL response with H-2-reconstituted membranes, but at levels much lower than that obtained using intact membranes. It was found that activity comparable to that of intact membranes is obtained if an actin-containing matrix isolated from tumor cell plasma membranes was included during formation of the liposomes (Herrmann & Mescher, 1981a). This matrix
containing actin and several other proteins is localized at the inner face of the plasma membrane where it appears to form a membrane skeleton (Mescher et al., 1981). We have, therefore, called these proteins cytoskeletal proteins (CP).

The functional role of these CP described here and elsewhere (Mescher et al., 1981) is not known. They may have a structural role, serving to stabilize the membrane bilayer. When used in reconstitution studies, they do increase overall size and heterogeneity of the size distribution of vesicles (Herrmann & Mescher, 1981b). The location of the matrix also makes these proteins likely candidates for mediating interactions between transmembrane proteins and the filament systems of the cytoskeleton, particularly the microfilament system. The CP may serve as anchorage point in the inner membrane surface for components of the cytoskeletal system.

Because of the enhanced recognition of H-2 antigens in the secondary stimulation assay caused by the CP, we have isolated a similar cytoskeletal matrix from our control cells for incorporation into liposomes. We are using an operational definition for CP, referring to the structures left after extraction of purified plasma membranes with the non-ionic detergent NP-40.

Others have operationally defined CP as those structures left after extraction of whole cells with specific (usually non-ionic) detergents (Brown et al., 1976;
Osborn & Weber, 1977; Small & Celis, 1978; Schliwa & Blerkom, 1981; Schliwa et al., 1981; Penman et al., 1982). Ultrastructural studies as well as biochemical and immunochemical analyses indicated that, besides specific cytoplasmic filaments, many other internal cellular components such as nucleic acids, different organelles, and some membrane proteins were retained after whole cell extraction (Fulton et al., 1980; Cervera et al., 1981; Fulton et al., 1981; Penman et al., 1982). The use of purified plasma membranes greatly reduces the contamination of the CP by these other components. However, a cell-surface glycoprotein, 5'-nucleotidase, remains associated with the isolated matrix (Herrmann & Mescher, 1981b).

Our major protein components coincide very closely to those previously reported for plasma membrane CP (Mescher et al., 1981). No effort was made at further characterization, and no attempt was made to detect any 5'-nucleotidase activity in our CP.

A problem with some protease activity was encountered, but the inclusion of a dounce homogenization step in the isolation procedure appeared to have eliminated that activity (Figure 4, lanes 6 and 7). This suggests that the protease was a soluble protein retained by incomplete solubilization of the pellet.
**Liposome characterization.** Liposomes, produced from lipids, constitute a family of vesicles exhibiting either multilamellar or unilamellar membrane structures. Liposomes prepared by detergent dialysis tend to be small unilamellar vesicles 100-200 nm in diameter (Kagawa & Racker, 1971).

Herrmann and Mescher (1981b) have extensively characterized their liposomes formed by detergent dialysis of purified H-2K\(^k\) antigen, CP from P815 cells, and lipids obtained from P815 tumor cells by chloroform:methanol (2:1) extraction. Our results closely agree with theirs. For example, they observed 30% incorporation of the H-2K\(^k\) into vesicles in the absence of CP, but 50% when the CP were included. We report 32% incorporation of H-2K\(^b\) in the absence of CP and 59% incorporation in their presence. Likewise, the sucrose densities to which their liposomes floated are comparable to ours, taking into account their higher lipid:protein ratio (11:1, wt:wt). In both studies, inclusion of CP during liposome formation resulted in formation of vesicles of higher density and a more heterogeneous distribution on the sucrose gradient. Electron microscopy showed a larger size and a more irregular shape for CP containing vesicles (Herrmann & Mescher, 1981b). Also note that fraction 3 in Figure 6A of our CP-free vesicles contained a significant amount of \(^{125}\text{I}-\text{labeled H-2K}^b\) but no \(^{14}\text{C}-\text{labeled cholesterol.}\)

In the above mentioned study (Herrmann & Mescher, 1981b), there was a hint of separation of H-2K\(^k\) into two peaks on
the sucrose gradient when reconstituted in the absence of CP, but neither cholesterol nor lipid was monitored.

The accessibility of the H-2 antigen on the liposome surface is an important parameter for lymphocyte recognition of the antigen. In the study by Herrmann and Mescher (1981b), papain treatment of liposomes prepared with and without CP demonstrated that about 70-85% of the H-2K\textsuperscript{k} antigen was accessible to the enzyme in both cases. We observed only 40% of the incorporated H-2K\textsuperscript{b} antigen being cleaved and only in the vesicles containing CP. However, the protease used was Pronase E, a nonspecific mixture of proteases from *Streptomyces griseus*, so a direct comparison of the results is not possible. Pronase preparations are known to be autoproteolytic and complete digestion may not be achieved (Metzler, 1977). Our work does suggest that the CP aid in the vectorial reconstitution of H-2K\textsuperscript{b} into vesicles. In contrast, several previous reports have rigorously shown that incorporation of MHC antigens into liposomes without CP by detergent dialysis results in the preferential orientation of the antigens on the outer surface (Engelhard et al., 1978; Turner & Sanderson, 1978; Willoughby et al., 1978; Littman et al., 1979; Curman et al., 1980).

**Inhibition of conjugate formation: the method.** Liposomes containing purified MHC antigens have been used to obtain an understanding of the structure of the protein in a lipid
bilayer, to test hypotheses concerning the interaction of the protein with the native plasma membrane, and to examine the parameters affecting the functional activity of the MHC antigens in the liposomes (reviewed in Strominger et al., 1981; Burakoff & Mescher, 1982). A great deal of successful effort has been reported in the use of reconstituted vesicles containing MHC products in the stimulation of T lymphocyte proliferation or cytotoxic function (see above). However, the use of such liposomes to examine events of the effector stage of immune responses is at a less advanced stage, partly because of the lack of a reliable, reproducible assay. Several groups have tried to demonstrate specific blocking of allogeneic CTL by membranes bearing the appropriate alloantigen using $^{51}$Cr-labeled target cells and measuring $^{51}$Cr release after 4 hr incubations. Some have reported specific blocking at high membrane concentrations (Wagner & Boyle, 1972; Bonavida, 1974; Gorezynski et al., 1975; Linna et al., 1978) while others have found none (Brunner et al., 1968; Berke & Amos, 1973; Sendo et al., 1974; Todd et al., 1975; Gilmer, unpublished observations). Nonspecific inhibition effects were usually seen at the high membrane concentrations. Moreover, in cases where specific inhibition was seen, there was often considerable difficulty in getting quantitatively reproducible inhibition from one membrane preparation to another.
The standard 4 hr $^{51}$Cr-release assay used above is a short term assay relative to the 5 day secondary stimulation assay, so the composition of vesicles is less likely to be altered in the course of the assay. In both cases the MHC antigens are recognized by a cell, initiating a measurable biological response. But the secondary stimulation assay involves complex interactions on several levels between different cell populations. The CTL-target cell interaction is a simpler system to examine and, therefore, easier to use and interpret in studying parameters affecting the functional activity of H-2 antigens.

The 4 hr $^{51}$Cr-release assay contains a major flaw which has probably led to some of the problems described above. In competition for CTL binding, intact target cells are favored over membranes, thus minimizing blocking by the membranes. At least three factors contribute to this flaw. Generally, the effector and target cells are brought together for interaction by settling or centrifugating to the bottom of a tube, greatly concentrating the cells but not the smaller, less dense membrane vesicles. CTL recycling also permits the lysis of more than one target cell by individual uninhibited CTL during the 4 hr assay. Lastly, $^{51}$Cr release from target cells involves all the discrete stages of CTL-target cell interaction: recognition and binding (conjugate formation), programming for lysis, and lysis (Martz, 1977). Vesicles would influence only the
recognition and binding stage. If this stage were not rate-limiting, inhibition by liposomes could be minimal.

Inhibition at the recognition stage has been assessed by microscopic counting of lymphocyte-target cell conjugates (Gilmer et al., 1978; Whisnant et al., 1978; Hale et al., 1981). Membranes bearing the appropriate H-2 antigens were found reproducibly to inhibit conjugate formation. This assay circumvents the recycling of the CTL by keeping the conjugates that have already formed at a temperature at which they will not readily dissociate nor will new conjugates readily form. The first stage most likely to be influenced by vesicles, the recognition and binding of target cells by CTL, is examined directly, eliminating the question of what is the rate-limiting step for the overall process. The membranes used in the above studies were large (300-500 nm, as in Lemonnier et al., 1978; Gilmer, 1982) compared to liposomes (50-200 nm as formed by various methods) and a higher than usual centrifugation speed (1325 x g instead of 300 x g), as well as a small volume (35 μl), reduced the problem of selective concentration of the cells over membranes. For these reasons it was concluded that this assay had more potential for the development of a reproducible protocol to examine the parameters influencing the recognition of reconstituted H-2 antigens by CTL.

The inhibition of T cell-target cell conjugate formation assay was further modified to increase the
likelihood of successful inhibition by appropriately constructed liposomes. To further reduce the possibility of T cell recycling, Ca\(^{+2}\) was removed from the reaction medium by eliminating it from the incubation buffer and by including EGTA, a Ca\(^{+2}\) chelator. Calcium is required for the lytic phase of the CTL-target cell interaction, and without it, the T cell will not program the target cell for lysis and dissociate (Golstein & Smith, 1976 & 1977; Plaut et al., 1976). The other major modification was based on the report of Elliott et al. (1979) in which radiolabeled membrane vesicles prepared by nitrogen cavitation of allogeneic stimulator cells were shown to specifically bind to T cell blasts. Vesicles from syngeneic spleen and lymph node cells did not bind as effectively. The binding appears to be temperature dependent, with most of the reaction occurring in the first hr of incubation at 37\(^{\circ}\) C.

Consequently, T cells were incubated with liposomes for one hour at 37\(^{\circ}\) C before combining them with the target cells.

An additional factor in the experimental design may best be described in terms of enzyme kinetics. The standard rate vs. substrate concentration curve shows a hyperbolic increase up to some point at which a plateau is reached. Beyond that point the enzyme is saturated and the rate of reaction is insensitive to the substrate concentration. Competitive inhibitors act only to increase the apparent Km for the substrate and have no effect on the maximum
velocity. Therefore, any competitive inhibitors added at a substrate concentration beyond the saturation point may have little if any influence on the rate because of the high substrate concentration. To optimize the possibility of observing inhibition with increasing inhibitor concentrations, a constant substrate concentration corresponding to about 50% of the maximum velocity, i.e., the Km, should be chosen. Berke et al. (1975) have presented data for CTL-TC conjugates analogous to the enzyme-substrate formulation using the same cell system that we used. They plotted the % T cells (BALB/c anti-EL4 peritoneal exudate cytotoxic lymphocytes) in conjugation (analogous to reaction rate) vs. the number of EL4 TC (analogous to substrate concentration) and showed a plateau of about 35% conjugate formation at an effector:target ratio of less than or equal to 1:1 (effectors = CTL). One half that % conjugation was observed at an effector:target ratio of about 2:1, and that ratio was chosen for our experiments.

The effectiveness of the redesigned assay in measuring the functional activity of reconstituted H-2K\textsuperscript{b} can be judged by the low amount of H-2K\textsuperscript{b} required (5 ng or 80 fmol) to yield 50% of the maximum observed inhibition of conjugate formation (Figure 7). This amount of H-2K\textsuperscript{b} is 30- to 90-fold less than the amount necessary (150-450 ng) to stimulate a secondary cell-mediated lysis response in vitro (Herrmann & Mescher, 1981a). Reproducible experiments were
attained as long as freshly prepared vesicles were used in the assay.

The question of the specificity of the observed inhibition is of great importance. Our vesicles containing H2Kb have been found to inhibit conjugate formation between H-2b targets and anti-H-2b effectors while vesicles containing H-2K do not. This could be due to specific inhibition or could result from greater non-specific inhibition by the H-2Kb liposome preparation. These alternatives are usually distinguished by performing double reciprocal experiments in which the same two liposome preparations would also be tested with H-2K targets and anti-H-2K effectors. However, non-specific inhibition would most probably be caused by simple mechanical interference preventing effective CTL-TC interaction. Such interference would also be expected to have some influence on the non-specific binding of cells since non-specific binding forces are also subject to mechanical interference. Neither of the vesicles preparations had any effect on the non-specific levels of binding even at antigen concentrations at which both preparations inhibited the specific conjugate formation (data not shown). This suggests that the observed inhibition was, in fact, specific at the liposome concentrations used, and also supports the likelihood that the inhibition by H-2K liposomes at higher concentrations was a cross reactive reaction of the type
noted elsewhere with intact cells (Schick & Berke, 1979). In addition, the effects of non-specific mechanical interference would not be expected to plateau at a level higher than non-specific binding, as did our inhibition, but progressively to reduce conjugate formation to zero.

Inhibition of conjugate formation by vesicles never did approach the limiting value (i.e., that observed with non-specific target cells), perhaps because there were T cells of such high affinity that vesicles containing reconstituted antigen could not compete with target cells. Alternatively, some of the allogeneically primed CTL might have been directed toward H-2D\textsuperscript{b} and, therefore, could not be inhibited by membrane-reconstituted H-2K\textsuperscript{b}. Other evidence suggests that these allogeneically primed T cells are primarily directed toward H-2K\textsuperscript{b} (Gilmer \textit{et al.}, 1984). Use of cloned CTL might help distinguish between these two alternatives.

The same inhibition of conjugate assay has been used to examine the biological activity of an H-2 preparation obtained from lentil lectin affinity chromatography of purified plasma membranes (Hale \textit{et al.}, 1981). Since the H-2 was not pure, 1250 ng protein was required in order to observe significant inhibition.

In comparison with earlier work, 180 ng purified plasma membranes (based on the revised yield of plasma membranes in Gilmer, 1982) in a 7 μl sample was required to inhibit
conjugate formation (Gilmer et al., 1978). Now using 5 ng of H-2K\textsubscript{b} plus 23 ng CP (or 28 ng total protein), we observed similar inhibition. This is 6-fold less total protein using the reconstituted vesicles to inhibit conjugate formation rather than the plasma membrane vesicles.

There are 0.064 ng of H-2K\textsubscript{b} (using 2 \times 10^5 molecules/target cell) on 3.2 \times 10^3 target cells (in 7 \mu l of the analyzed sample). Therefore, we required 80-fold more H-2K\textsubscript{b} in vesicles than is present on the target cells to observe 50\% inhibition. This decrease in efficiency of the vesicles over the cells could be attributed to any one of the following factors: 1) only partial vectorial orientation of the H-2K\textsubscript{b} in the vesicles (Figures 6C and 6D), 2) the absolute purity of the H-2K\textsubscript{b} (Figure 5), 3) the difference in efficiency of vesicles and whole cells binding specifically to the CTL (Herrmann & Mescher, 1981b), and 4) a suboptimal lipid composition, either for incorporation of H-2K\textsubscript{b} into vesicles or for display of the antigens to the CTL.

It is interesting to speculate regarding the role the CP may have had in achieving inhibition by H-2 in liposomes. Hollander et al. (1979), from whom we obtained the lipid composition of our liposomes, succeeded in attaining direct lysis of antigen-containing liposomes by CTL only when a human eye muscle protein preparation was included in the liposomes. Their results were not reproducible, however,
possibly due to the heterogeneity of the source of eye muscle protein. In a followup report, Mehdi et al. (1980) found that the appearance of the vesicles, as examined by electron microscopy, varied from one preparation to another. Effective lysis appeared to correlate with the presence of predominantly large vesicles having a lumpy texture.

Seemingly related is the previously cited work (Herrmann & Mescher, 1981a) in which a more effective secondary CTL response was stimulated by liposomes with CP incorporated. Again, the CP produced larger, more irregularly shaped liposomes when examined by electron microscopy (Herrmann & Mescher, 1981b).

CP could be involved in the recognition of the H-2 antigens at two levels. By physically increasing the size of the liposomes, both the surface area available for interaction with T cells and the subsequent potential valency of the liposome would be increased. Alternatively, upon addition of cytoskeletal components, MHC-cytoskeletal interactions necessary for recognition and biological function of the membrane antigens may be introduced. This may include enhancement of the vectorial orientation of the antigens in the membrane.

The interaction of MHC antigens with cytoskeletal proteins in vivo is suggested by several lines of evidence in both the murine and human systems. Using double immunofluorescence microscopy, the arrangement of the H-2K
and -D antigens, and HLA-A and -B antigens, on the cell surface was shown to coincide with the arrangement of submembraneous cytoskeletal proteins in a number of cell types, including lymphocytes (Bourguignon et al., 1978; Huet et al., 1980). In contrast, the same technique has also indicated that the microtubule organizing center in essentially all conjugated CTL, but not in the conjugated target cells, were oriented towards the intercellular contact site (Geiger et al., 1982). Similarly, the area of contact of CTL (but not of the TC) with their target cell has also been shown to be an area of high motility and polarization of actin, but not of myosin (Ryser et al., 1982). The recognition event itself seems to have more than one step, one of which is divalent cation-independent and sensitive to cytochalasin A (MacLennan & Golstein, 1978). A more extensive study of the effects of cytochalasins from 5 known classes showed that all the cytochalasins tested blocked the recognition of TC by CTL, none blocked the TC lysis stage, and all but cytochalasin A blocked Ca^{2+}-dependent events of the lethal hit stage (Golstein et al., 1978). In these last two studies, the cytochalasins were added to both the CTL and TC, so it is not possible to assess whether the essential cytoskeletal components were in the effector or target cell populations. Electron microscopic examination of the lymphocyte population in the
second study (Golstein et al., 1978) did show disruption of the microfilaments.

On the molecular level, a specific association of actin with H-2K and -D molecules has been observed in shed fragments of membrane (Koch & Smith, 1978), in the immunoprecipitation of the detergent-solubilized antigens by alloantisera (Gilmer, 1982), and in the mcAb affinity purification of the detergent-solubilized antigens (Figard & Gilmer, unpublished observations). Consistent with cytoskeletal restraint of the mobility of HLA-A and -B antigens is the observation that the lateral mobility of HLA antigens in the plane of human leukocyte cell membranes is retarded at least 13-fold relative to that of the lipid molecules in the same membrane, as determined by fluorescence photobleach recovery experiments (Petty et al., 1980). In other experiments, the HLA-A2 antigen was labeled specifically in its carboxy-terminal intracellular region with a fluorescent probe and a change in the fluorescence signal of the reporter group was observed upon in vitro recombination with lymphoblastoid CP (Pober et al., 1981). The exact nature of the cytoskeletal-MHC antigen interactions and their role in CTL activity remains to be investigated.

Since the amount of purified H-2 required for inhibition of T cell-target cell conjugates is relatively small, this assay can be used to test how parameters such as
the lipid composition, membrane fluidity, surface charge, the subcellular source of the antigens, and the surface density of the antigens affect conjugate formation. In this way the parameters that are critical for H-2 recognition by CTL can be evaluated.

**The role of sialic acid in the recognition of H-2 by CTL.**

In order to assess the influence of the carbohydrate moieties on the recognition of H-2 antigens by CTL, the assay discussed in detail above was developed. This assay consists of a greatly simplified system which uniquely examines only the recognition event and only the recognition of the H-2 antigen in the liposome membrane. We have also described in detail the selection of a source of H-2 antigen that provides an antigen differing from the native molecule, presumably, only by the total loss of terminal sialic acid residues from its carbohydrate side chains, although rigorous proof for the identity of the remaining sugars is lacking.

The failure to detect any significant difference in inhibition with the two forms of H-2 antigens in our assay (Figure 8) is significant, as this has not been unequivocally demonstrated in the past (cf. Introduction). This strongly suggests that the sialic acid does not play a direct role in masking any antigenic determinants, nor does it appreciably alter the protein conformation of any determinants recognized by T cells. Thus, recognition by
CTL is unaltered by the carbohydrate change, in agreement with some other reports on this subject (MacDonald & Cerottini, 1979; Hooghe et al., 1984). It had been previously shown that the carbohydrates have no influence on the recognition of H-2 antigens by antibodies (Shimada et al., 1970; Nathenson & Cullen, 1974). Further alterations of the carbohydrate, either by the selection of more variants for sources from which to purify antigen, or by enzymatic modification of already purified antigens, should definitively demonstrate whether the carbohydrate has any part in the recognition event in CTL killing.

Given the well known distinction between CTL-target cell binding and subsequent lysis of the target cell, it is possible that the carbohydrate of the H-2 antigens may influence the killing of the target cell without being involved in the recognition event. In a recent article, Nose and Wigzell (1983) demonstrated that the depletion of the asparagine-linked carbohydrate chains on monoclonal, hapten-specific mouse IgG2b antibodies had no effect on the fine antigen-binding reactivity and protein A binding capacity. However, they lost the ability to activate complement, to bind to Fc receptors on macrophages, and to induce antibody-dependent cellular cytotoxicity. Moreover, antigen-antibody complexes of carbohydrate-deficient antibodies were not eliminated rapidly from the circulation. Thus, the carbohydrate side chains have no influence in
antigen binding (recognition), but have a very profound impact on the biological activity of the antibodies. Several other proteins have their biological function regulated by their degree of sialylation (reviewed in Reutter et al., 1982; Schauer, 1982). The same may be true in the case of the carbohydrate of the H-2 antigens.
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