***Brief Definitive Report***

# CARBOHYDRATE MOIETIES OF MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I ALLOANTIGENS ARE NOT REQUIRED FOR THEIR RECOGNITION BY T LYMPHOCYTES

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Cytotoxic T lymphocytes (CTL) have been implicated as effectors in allograft rejection and in control of viral infection. Induction of a cytolytic response requires T cell recognition of foreign major histocompatibility complex (MHC) class I glycoproteins, or syngeneic MHC antigens along with a foreign (i.e., viral) protein. This recognition process leads to proliferation and differentiation of precursor cells to effector CTL capable of lysing target cells that bear the same antigens. While class I oligosaccharide residues do not contribute to serologic determinants (1–4) their role in T cell recognition has remained unclear. Modification of the carbohydrates of membrane glycoproteins on intact stimulating or target cells has been observed to inhibit (1, 5, 6), stimulate (7, 8), or have no effect (2, 6, 9) on T cell responses. In all of these cases, it is difficult to conclude whether the observed effects were due to altered MHC antigen oligosaccharides, since modifications could not be limited to the surface glycoprotein of interest, and the extent of modification could not be readily determined.

Class I antigens of the murine MHC (H-2) are polymorphic two-chain cell surface glycoproteins consisting of a glycosylated transmembrane heavy chain ($M_r$ 40,000–50,000) noncovalently associated with $\beta_2$-microglobulin, an invariant, nonglycosylated polypeptide ($M_r$ 11,500). Amino acid and DNA sequence data suggest that the extracellular portion of the heavy chain can be divided into three globular domains of ~91 amino acids each ($\alpha_1$, $\alpha_2$, and $\alpha_3$). Determinants recognized by T cells appear to reside in the $\alpha_1$ and $\alpha_2$ domains (10), regions of maximal amino acid variability (11). Glycosyl moieties are also present in these regions, *N*-linked to asparagine residues at positions 86 and 176. A third glycan attachment site in $K^d$, $D^b$, and $L^d$ antigens is at residue 256 (11).

Purified class I alloantigen in artificial membranes (liposomes) can stimulate a CTL response of comparable magnitude to that obtained using intact stimulating cells when added to in vitro cultures of spleen cells from mice previously primed to the same alloantigen (12). The magnitude of the response to the liposomes provides a direct measure of the effectiveness of antigen recognition by primed

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precursor CTL (pCTL), provided that an optimum amount of interleukin-containing supernatant is added (12). This approach has made it possible to examine the role of the carbohydrate moieties in class I MHC recognition.

## Materials and Methods

*Mice and Tumor Cells.* (BALB/c × DBA/2)$F_1$ ($CD2F_1$) (H-$2^d$) mice were purchased from Cumberland View Farms, Clinton, TN. (AKR × DBA/2)$F_1$ ($AKD2F_1$) (H-$2^{kxd}$) and A/J (H-$2^a$) were purchased from The Jackson Laboratory, Bar Harbor, ME. RDM-4 (H-$2^k$), an AKR lymphoma, and P815 (H-$2^d$), a mastocytoma of DBA/2 origin, were maintained by intraperitoneal passage as ascites in $AKD2F_1$ mice.

*Purification of Native and Radiolabelled H-2K$^k$.* H-2K$^k$ was purified from RDM-4 ascites cell detergent lysates by affinity chromatography on an 11-4.1 monoclonal antibody (mAb) column (12) with a yield of 0.9 mg H-2K$^k$ per $10^{10}$ cells. Internally radiolabeled H-2K$^k$ was purified from RDM-4 cells cultured in glucose- or methionine-deficient RPMI 1640 (Gibco Laboratories, Grand Island, NY) with [$^3$H]mannose or [$^{35}$S]methionine, and 5% dialyzed fetal calf serum. $^{125}$I-H-2K$^k$ was prepared by reacting 10 μg purified antigen with 0.5 mCi Na$^{125}$I and two Iodobeads (Pierce Chemical Corp., Rockford, IL) in 0.15 ml 0.5% deoxycholate for 30 min at 4°C, and isolated by Sephadex G-25 chromatography and dialysis, yielding a product with a specific activity of 400 cpm/ng protein.

*Preparation of Liposomes and Deglycosylated H-2K$^k$.* Purified H-2K$^k$ was incorporated into unilamellar liposomes by mixing with lipid (1 μg H-2K$^k$ per 10 nmol phospholipid) in 0.5% deoxycholate, followed by dialysis to remove the detergent (12). Liposomes were harvested by centrifugation at 100,000 *g* for 60 min, and resuspended in 33 mM EDTA, 10 mM Tris maleate, 170 mM NaCl, pH 6.1. Liposomes containing 5 μg H-2K$^k$ were subjected to either limited (1 U enzyme for 4 h) or exhaustive (4 U for 21 h) endoglycosidase F treatment (endo F) (EC-3.2.1; New England Nuclear, Boston, MA) at 37°C. Controls without enzyme were incubated in parallel at 4°C and 37°C. In some experiments, liposomes were reisolated from the enzymatic reaction mixture by centrifugation. All H-2K$^k$ preparations were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 5–15% slab gels.

*Effector CTL Generation and Cytotoxicity.* Liposomes were added to responder splenocytes from $CD2F_1$ mice primed 2–4 mo previously by intraperitoneal injection of $2 \times 10^7$ RDM-4 cells, and cultured in 2 ml of medium with $7 \times 10^6$ cells/well (Linbro Chemical Co., Hamden, CT) at 37°C and 5% $CO_2$. An optimal concentration of an $(NH_4)_2SO_4$ fraction of supernatant fluid from rat spleen cells incubated with concanavalin A (12) was added at 22 h of culture. After 5 d, the cultures were assayed for cytotoxicity at several effector/target cell ratios by triplicate determination of the $^{51}$Cr released in 4 h from $1.5 \times 10^4$ labelled RDM-4 target cells incubated at 37°C. Medium was RPMI 1640 supplemented as previously described (12).

## Results

Endo F was used to obtain deglycosylated H-2K$^k$. This enzyme cleaves high-mannose and complex-type asparagine-linked glycans, leaving a single *N*-acetylglucosamine residue attached to the protein (13). The glycan-bearing heavy chain of native H-2K$^k$ is of 48,000 $M_r$ when examined by SDS-PAGE and autoradiography (Fig. 1, lane 1). In contrast, three heavy chain products were found when H-2K$^k$ was incorporated into liposomes and subjected to limited endo F treatment (Fig. 1, lane 2). These had $M_r$ of 48,000, corresponding to native H-2K$^k$, 45,000, and 42,000. While the 48,000 and 45,000 $M_r$ products were bound by lentil lectin–Sepharose, the 42,000 product was not (data not shown). This indicated that the 45,000 $M_r$ product retained one of the two original carbohydrate moieties, and that the 42,000 product was fully deglycosylated. Tunicamycin treatment of cells to inhibit lipid-dependent protein glycosylation yields a mixture of H-2K$^k$ heavy chain products, including a fully deglycosylated species with an $M_r$ 6,000 smaller than native antigen (3).

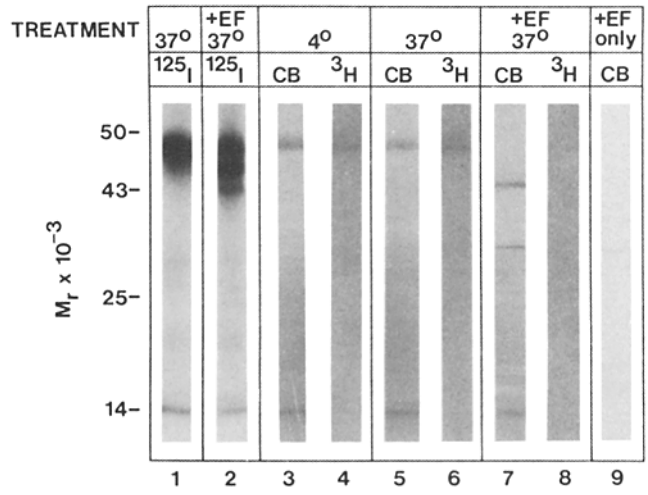


FIGURE 1. Deglycosylation of H-2K$^k$. Purified H-2K$^k$ in liposomes was subjected to limited or complete deglycosylation by endo F (*EF*) and analyzed by SDS-PAGE. Protein bands were visualized by autoradiography ($^{125}I$) or staining with Coomassie Blue (*CB*) and fluorography ($^3H$). Liposomes containing 5 μg of H-2K$^k$ and $5 \times 10^5$ cpm in 0.05 ml underwent the following endo F treatments: lane 1, no enzyme, 4 h, 37°C; lane 2, 1 U enzyme for 4 h, 37°C; lanes 3 and 4, no enzyme, 21 h incubation at 4°C; lanes 5 and 6, no enzyme, 21 h, 37°C; lanes 7 and 8, 4 U, 21 h, 37°C; lane 9, 1 U endo F without liposomes.

Extensive endo F treatment of liposomes bearing H-2K$^k$ resulted in complete conversion of heavy chains to the 42,000 $M_r$ product, as demonstrated by Coomassie Blue staining after SDS-PAGE (Fig. 1, lane 7). Incubation of liposomes for the same length of time at either 4°C (lane 3) or 37°C (lane 5) in the absence of enzyme resulted in no change in the $M_r$ of heavy chains. The absence of oligosaccharides in the 42,000 $M_r$ product was confirmed by examination of H-2K$^k$ biosynthetically labelled with [$^3$H]mannose. No detectable radioactivity remained associated with the 42,000 $M_r$ product resulting from endo F treatment (Fig. 1, lane 8), while controls retained the labelled glycans (lanes 4 and 6). The observation that all heavy chains are deglycosylated confirms that the antigen is asymmetrically oriented on the external face of these sealed unilamellar vesicles.

Treatment with endo F resulted in no change in the $M_r$ of $\beta_2$-microglobulin (Fig. 1), nor in its association with heavy chain, as assessed by radioactivity recovered from the bands in gels of deglycosylated $^{125}$I-H-2K$^k$. Serological activity detected by the 11-4.1 mAb was also unaffected, since immunoprecipitation of deglycosylated [$^{35}$S]methionine-labelled H-2K$^k$ was 92% that of native labelled antigen (data not shown). These observations are consistent with studies of H-2 and HLA class I antigens indicating that carbohydrate groups do not affect heavy and light chain association (4), or contribute to alloantigenic serologic determinants (1–4). In fact, blocking glycosylation does not prevent synthesis and expression of serologically active antigen at the cell surface (1, 4).

The ability of pCTL to recognize and respond to native or deglycosylated H-2K$^k$ on liposomes was assessed by measuring their maturation to effector CTL capable of lysing antigen-bearing target cells. Addition to cultures of liposomes bearing either native or deglycosylated H-2K$^k$ resulted in strong and comparable responses (Fig. 2*A*). No cytolytic response was obtained in the absence of antigen-

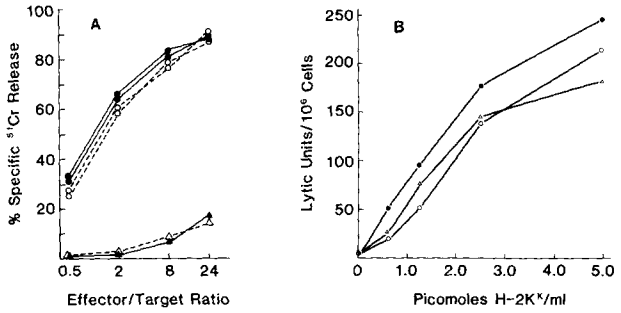


FIGURE 2. *A*, Precursor CTL recognition of native and deglycosylated H-2K$^k$. Immune recognition was assessed by the development of cytotoxicity towards $^{51}$Cr-labelled RDM-4 target cells in cultures of primed CD2F$_1$ responder splenocytes incubated for 5 d with: (△) medium only; (○) 0.125 μg/ml native H-2K$^k$ in liposomes; (●) 0.125 μg/ml deglycosylated H-2K$^k$ in liposomes; (▲) 0.75 U/ml endo F. The native and deglycosylated H-2K$^k$-bearing liposome preparations added to these cultures were analyzed in Fig. 1. Percent specific $^{51}$Cr release = 100 × (experimental − spontaneous $^{51}$Cr release)/(total releasable − spontaneous $^{51}$Cr release). Spontaneous release was <18% for all experiments. *B*, Generation of effector CTL by increasing doses of native and deglycosylated H-2K$^k$. Liposomes were subjected to exhaustive endo F treatment (4 U, 21 h, 37°C), or parallel control incubations without enzyme at 4°C or 37°C; reisolated from incubation mixtures by centrifugation, and suspended in medium for culture. A trace of $^{125}$I-H-2K$^k$ was included in the liposome preparation to permit antigen quantitation. Endo F–treated liposomes were confirmed to bear only deglycosylated H-2K$^k$ by SDS-PAGE and autoradiography, while incubation in the absence of enzyme resulted in no change in the $M_r$ of the heavy chain. Cytotoxicity was measured after CD2F$_1$ responder splenocytes were cultured for 5 d with liposomes bearing: (●) deglycosylated H-2K$^k$; (△) native H-2K$^k$ (pretreated at 4°C); (○) native H-2K$^k$ (pretreated at 37°C). 1 pmol of native H-2K$^k$ is equivalent to 0.048 μg. Lytic units provide an estimate of the relative number of effector CTL in a population. One lytic unit is defined as the number of cultured cells required to lyse 50% of the target cells in the 4-h $^{51}$Cr release assay.

bearing liposomes, nor after addition of endo F alone to cells in culture (Fig. 2*A*). Furthermore, neither the presence (Fig. 2*A*) nor the absence (Fig. 2*B*) of the enzyme in cultures with liposomes affected the level of response obtained to either native or deglycosylated antigen.

The response to native and deglycosylated H-2K$^k$ was examined over a wide range of antigen concentrations to rule out the possibility that a small amount of native H-2K$^k$ remaining in the endo F–treated samples might account for the observed stimulation. The deglycosylated antigen was found to stimulate somewhat more efficiently over the entire dose range for the response (Fig. 2*B*). This augmentation was observed with four independent preparations, and may result from decreased repulsion between treated liposomes and negatively charged surfaces of pCTL. In fact, removal of negatively charged sialic acid by neuraminidase increases target cell sensitivity to CTL (7, 8). Stability of the H-2K$^k$ molecule in either its native or deglycosylated state is underscored by maintenance of immunogenicity despite overnight incubation at 37°C.

To rule out possible nonspecific mitogenic effects of the deglycosylated class I antigen, the specificity of CTL induction and of the resulting effector cells was examined (data not shown). When splenocytes from A/J (H-2K$^k$) mice previously immunized with P815 (H-2K$^d$) cells were cultured for 5 d in the presence of plasma membranes from P815 cells, effector CTL were generated that lysed P815 but not RDM-4 targets. Culture of the same cells with liposomes bearing

either native or deglycosylated H-2K$^k$ resulted in no response using either tumor cell target. These liposomes, at the same and lower concentrations, stimulated a strong response by CD2F$_1$ spleen cells, and the resulting effectors were specific for target cells bearing the appropriate H-2K$^k$ alloantigen.

## Discussion

Liposomes bearing completely deglycosylated H-2K$^k$ have been shown in this report to trigger the maturation of precursor to effector CTL as effectively as liposomes with native antigen. It follows that class I oligosaccharide moieties do not contribute to alloantigenic determinants recognized by the majority of splenic pCTL. If a small number of pCTL can recognize determinants that include the carbohydrate, they constitute a minor portion of the allogeneic response. These results also show that induction of the cytolytic response does not require non–antigen specific interaction of H-2K$^k$ glycan moieties with either the T cell receptor or accessory molecules. A role for carbohydrates in other accessory interactions during target cell binding or lysis cannot be ruled out.

Recently, site-directed mutagenesis was used to delete the oligosaccharide at position 86 in H-2L$^d$ by substituting the original asparagine with a lysine residue, which is not glycosylated (14). Mouse L cell transfectants expressed the mutant product and were recognized by H-2L$^d$-reactive CTL. However, a limited number of conclusions can be drawn from this report regarding the role of H-2 oligosaccharides in T cell recognition. Only one of the three carbohydrate residues present in H-2L$^d$ was removed. Further, as noted by the authors, mutagenesis not only removed the glycan at position 86, but substituted another amino acid, which may affect conformational changes unrelated to the absence of the oligosaccharide. Finally, while the use of liposomes allowed quantitative assessment of the functional effects of removing all the carbohydrate from native H-2K$^k$, the gene transfer results are qualitative, as they are complicated by varied levels of antigen expression on different transfectants and by assumptions regarding the effect this might have on cytolysis by CTL.

The use of purified MHC antigen in liposomes to assess structural requirements for T cell recognition provides several advantages over use of modified whole stimulator cells. The modification can be confined to the antigen of interest, and the extent of modification can be readily determined. Furthermore, the dose response to the antigen or its modified forms can be accurately quantitated. The importance of this is indicated by the observation that removal of >90% of the H-2 from the surface of allogeneic cells by papain treatment did not decrease the CTL recognition of these cells (15). Thus, modification or removal of >90% of a critical antigen structure on intact cells can appear to have no effect.

## Summary

The ability to generate specific cytotoxic responses using purified major histocompatibility complex (MHC) antigen in liposomes has made it possible to directly assess the importance of class I carbohydrate moieties in T cell recognition of alloantigen. Deglycosylation of affinity-purified H-2K$^k$ to yield a single glycan-free product did not alter the specificity, the magnitude, nor the dose range of the cytotoxic T lymphocyte (CTL) response to the class I antigen. It

can be concluded that carbohydrate moieties are not required to maintain the necessary conformation of the MHC protein, nor to interact with either the antigen-specific receptor or accessory proteins on precursor CTL.

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