

SELECTIVE TURNOVER AND SHEDDING OF H-2K AND
H-2D ANTIGENS IS CONTROLLED BY THE MAJOR
HISTOCOMPATIBILITY COMPLEX

Implications for H-2-Restricted Recognition*

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Genes mapping in the major histocompatibility complex (MHC)¹ regulate the cytotoxic response of murine lymphoid cells to foreign membrane-bound antigens. Cytotoxic T cells recognize viruses (1, 2), minor histocompatibility antigens (3, 4), and trinitrophenol (TNP) (5, 6) in association with H-2K and H-2D region gene products. This coordinate recognition of H-2 products and foreign antigens results in both qualitative and quantitative control of specific cytotoxic responses. Moreover, the magnitude of the cytotoxic response generated against H-2K- or H-2D-compatible targets in these systems appears to be influenced as well by genes mapping in the MHC. For example, spleen cells from H-2^k strains preferentially respond to TNP in association with H-2K^k and not H-2D^k, whereas TNP is recognized preferentially in association with H-2D^d and not H-2K^d by mice from several H-2^d strains (7, 8). Similar genetic control has been described in H-2-restricted viral-specific (9-11) and sex-linked antigen-specific (1, 2, 12) cytotoxicity systems. The basis for this genetically controlled quantitative variation in cytotoxic responses is unknown, but might involve structural variations between H-2K and H-2D antigens, regulatory Ir-like gene effects, or both.

We have shown previously (13) that cell surface I-A antigens were synthesized and shed rapidly by murine spleen cells, whereas I-E antigens had a much more prolonged membrane residence. These results raised the possibility that the rapid shedding of I-A antigens played a key role in the ability of allogeneic cells to stimulate a mixed lymphocyte reaction. Because TNP-modified H-2 antigens have been shown to be lost from cultured stimulator cells (14), we reasoned that the observed variations in responses associated with H-2K and H-2D gene products could be related to the cellular metabolism of H-2 antigens.

In this study we sought to determine whether the metabolism of H-2K and H-2D

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¹ Abbreviations used in this paper: MHC, major histocompatibility complex; NMS, normal mouse serum; PBS, phosphate-buffered saline; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; TNP, trinitrophenol.

membrane proteins present on murine spleen cells varied within the same haplotype or among H-2 haplotypes. H-2 antigens were found to be turned over and shed at different rates depending on the H-2K or H-2D allele examined. In addition, we found that a locus mapping to the left of the I-A subregion (toward the centromere) regulates the turnover of an H-2D antigen. These variations show a striking correlation with the observed genetic variations in quantitative cytotoxic responses to chemically modified or virus-infected syngeneic cells.

Materials and Methods

Mice. H-2-congenic and -recombinant mice from the breeding colony maintained at Yale University (New Haven, Conn.) were used.

Cell Preparation. Splens were dissociated into single cell suspensions in a loose fitting tissue grinder and washed twice in calcium- and magnesium-free phosphate-buffered saline (PBS, pH 7.2). The cells were then layered over Ficoll-Hypaque gradients and centrifuged at 1,500 g for 150 s. The mononuclear cells at the interface were removed and washed three times in PBS.

Cell Surface Radioiodination. Single cell suspensions were radioiodinated as previously detailed (13). All iodinations were performed in PBS at reactant concentrations of 0.7 μ M lactoperoxidase, 50 μ M H₂O₂, and 0.5 μ M Na ¹²⁵I or ¹³¹I (New England Nuclear, Boston, Mass.). Cell viability after labeling was always greater than 96%, as determined by the exclusion of diamine (Trypan) blue dye.

Cell Culture. The radiolabeled cells were resuspended in RPMI-1640 medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 5% heat-inactivated fetal calf serum (batch R267426; Grand Island Biological Co.), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM glutamine, at a concentration of 5×10^6 cells/ml. The conditions of the incubation, and of subsequent lysis and dialysis procedures, were as previously described (13).

Biosynthetic Labeling. For biosynthetic studies, cells were cultured in leucine-free, serum-free RPMI under conditions identical with those for the surface-labeled cells. At the beginning of each culture 250 μ Ci L-[4,5-³H]leucine (Amersham Corp., Arlington Heights, Ill.) was added to each 1-ml sample. For continuous incorporation experiments the cells were cultured in the presence of radioisotope for the full 30 h. In pulse-chase experiments the cells were cultured in the presence of [³H]leucine for 4 h, then washed twice, resuspended, and incubated in complete RPMI-1640 medium for the remainder of the culture.

Antisera. The alloantisera used in these studies were prepared by repeated immunization of lymph node and spleen cells between H-2-congenic and -recombinant strains. The specificities of these sera are as follows: (A.TL \times C3H.OL)_{F1} anti-C3H (anti-H-2K^b); (A.TL \times B10.A)_{F1} anti-B10.BR (anti-H-2D^b); (BALB.B \times A.AL)_{F1} anti-A.TL (anti-H-2K^b); (B10.D2 \times A.TH)_{F1} anti-A.SW (anti-H-2D^b); B10.S(9R) anti-B10.D2 (anti-H-2K^d, I-A^d, I-B^d, I-J^d, I-E^d); (C3H.OH \times A.SW)_{F1} anti-A.TH (anti-H-2D^d); (B10.A(4R) \times A/Sn)_{F1} anti-B10 (anti-H-2K^b, I-A^b); (B10.A(18R) \times A/Sn)_{F1} anti-B10 (anti-2H-2D^b). The italicized specificities denote that ¹²⁵I-labeled surface proteins of 45,000 dalton were bound by the antiserum and resolved by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE). Weak, cytotoxic titers against nonitalicized specificities (Ia) were present in two of the sera, but these antigens were not precipitated at dilutions of serum used. Although some of the anti-D region sera may contain antibody against the H-2L product as well as the H-2D product, no distinction between these products is made in this study.

Formalin-fixed Staphylococcus aureus Cowan Strain I. A stab culture of *S. aureus* Cowan strain I which possesses a receptor for mammalian IgG (15, 16), was maintained on slant cultures. Formalin-fixed *S. aureus* were prepared as previously described (13, 17). For the most recent experiments formalin fixed *S. aureus* were obtained from Calbiochem-Behring Corp., American Hoechst Corp. (San Diego, Calif.).

Immunoprecipitation. After high-speed centrifugation, 1-ml cell pellet and supernatant dialysates were divided into fractions for immunoprecipitation. Each 1-ml sample was incubated with 400 μ l formalin-fixed *S. aureus* for 1 h at 4°C to decrease the level of nonspecific binding in the subsequent precipitation. After centrifugation at 10,000 g for 10 min, the cleared supernates were apportioned for precipitation with the specific alloantisera and normal mouse

serum (NMS) controls. 5 μ l of antiserum were incubated with each sample for 20–24 h at 4°C, and then 40 μ l *S. aureus* were added to each sample for 10 min at 4°C. All immunoprecipitates were then washed with 0.05% Triton X-100/Tris until maximum specificity was achieved. Background NMS radioactivity was 15–25% of specific counts for all precipitations.

Polyacrylamide Gel Electrophoresis (PAGE). Immune complexes bound to *S. aureus* were eluted with SDS-PAGE solvent buffer (18) plus 15% (vol:vol) β -2 mercaptoethanol. Eluted polypeptides were resolved by discontinuous electrophoresis in polyacrylamide gels as previously described (18). 125 I-labeled murine IgM and ovalbumin were subject to coelectrophoresis with the 125 I-labeled membrane proteins to serve as internal markers. For SDS-PAGE analysis of [3 H]leucine-labeled samples, *N,N*-diallyl tartardiamide cross-linker was substituted for bis-acrylamide. After electrophoresis, gel slices were dissolved in 2% periodic acid for 20 h, diluted in 7 ml Aquasol scintillant (New England Nuclear), and assayed for radioactivity in a Beckman LS-230 scintillation counter.

Results

Cell Surface H-2K Antigens Are Shed Preferentially vs. H-2D Antigens on CBA/J Spleen Cells (Fig. 1). The preferential shedding of H-2K^k antigens from CBA/J spleen cells is shown in Fig. 1. Splenocytes were surface labeled by lactoperoxidase-catalyzed radioiodination (13) and incubated in tissue culture medium for 0–30 h. Portions were periodically removed, cell and medium supernatant fractions were separated by centrifugation, and the cell pellets were lysed in 0.5% Triton X-100 detergent. At the conclusion of the incubation, each cell lysate and supernatant fraction was divided for immunoprecipitation with anti-H-2K^k, anti-H-2D^k, or NMS. The soluble antigen-antibody complexes were then sedimented with whole *S. aureus*, and the pellets were washed until maximum specificity was achieved.

The rate of loss of cell surface H-2^k antigens was determined by comparing the radioiodine remaining in cell H-2^k antigens throughout the culture period (Fig. 1A). Whereas H-2D^k antigens were lost uniformly and slowly ($t_{1/2}$ = 30 h), H-2K^k antigen loss showed biphasic kinetics. Approximately 50% of radioiodinated H-2K^k was lost within 8 h ($t_{1/2}$ = 8 h), whereas subsequent H-2K^k loss was slow ($t_{1/2}$ = 30 h). During this culture interval cell viability decreased from 97% at 0 time to approximately 90% at 8 h and 80% at 30 h. Cell number was unchanged throughout.

Over the first 4 h, all specific H-2K^k radioactivity lost from the cell fraction could be recovered in culture supernate (Fig. 1B). Accumulation of iodinated H-2K^k antigens then began to plateau, and in fact actually diminished during the latter half of the incubation. Summation of cell plus supernatant H-2K^k activity thus illustrates the gradually declining level of total precipitable H-2K^k antigen. Radiolabeled H-2D^k antigens could not be detected in culture supernates at any time during the incubation. Thus, cell surface H-2K^k antigens are preferentially shed vs. H-2D^k antigens from cultured CBA/J spleen cells.

Shed H-2K^k Antigens Have Identical SDS-PAGE Mobility to Cell Surface H-2K^k Molecules (Fig. 2). SDS-PAGE analyses of the H-2 antigens isolated from cell lysate and supernatant fractions are shown in Fig. 2. Both cell surface H-2K^k and H-2D^k antigens show identical electrophoretograms, with over 80% of the specific iodine recovered in one band at 44,000 daltons and a minor band migrating close to the dye front. SDS-PAGE analysis of the H-2K^k and H-2D^k antigens at varying times throughout the incubations indicated unaltered mobilities for the labeled cell surface molecules. Likewise, H-2K^k antigens isolated from culture supernates after 8 h culture showed identical mobility with the cell surface molecules (Fig. 2B). Thus, gross proteolytic

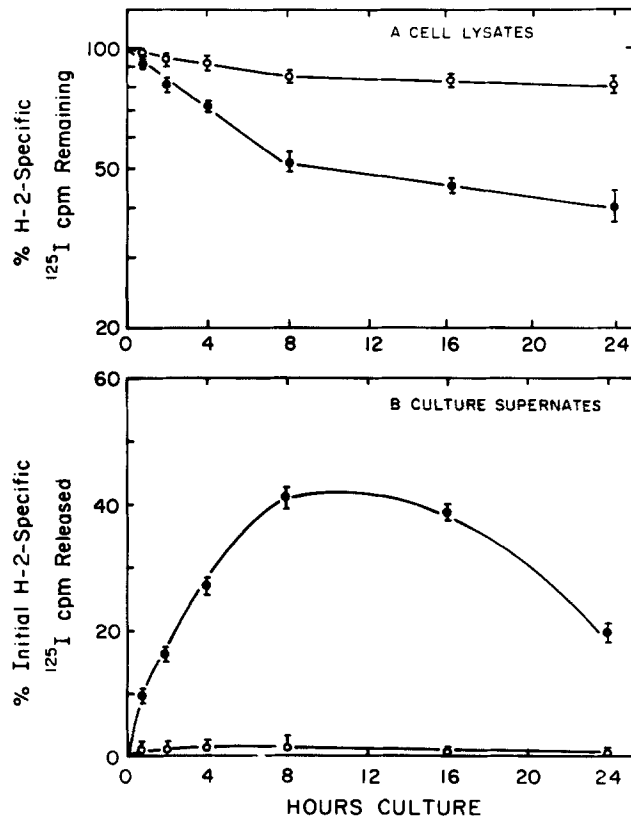


FIG. 1. Selective shedding of H-2K antigens by CBA/J spleen cells. Surface-iodinated spleen cells were cultured for 0-30 h. Samples were withdrawn and separated by centrifugation into cell pellet and supernatant fractions. Cell pellets were lysed, and after dialysis cell and supernatant dialysates were apportioned for precipitation with anti-H-2K^d, anti-H-2D^k, and NMS. Each point indicates experimental minus control counts per minute, and represents the mean result of seven experiments, with brackets showing the standard error. ●—●, H-2K; ○—○, H-2D. (A) analyses of cell lysates, with % counts per minute remaining at time X = specific counts per minute (time X) - control counts per minute (time X)/specific counts per minute (time 0) - control counts per minute (time 0) × 100. (B) Analyses of culture supernatant, with % counts per minute released at time X = supernatant specific counts per minute (time X) - supernatant control counts per minute/cell specific counts per minute (time 0) - cell control counts per minute (time 0) × 100.

cleavage did not appear to be a feature of the H-2K^k shedding process. SDS-PAGE analysis of NMS precipitates, and of anti-H-2D^k precipitates from culture supernates, resolved patterns indistinguishable from detector background.

Shed Cell Surface H-2K^k Molecules Are Replaced by the Cultured Cells at the Plasma Membrane (Fig. 3). Double-label experiments were performed to demonstrate that shed H-2K^k molecules were stoichiometrically replaced at the cell surface. Single cell suspension were radioiodinated with ¹²⁵I and placed into tissue culture for 8 h. The cells were then centrifuged, washed, and relabeled with ¹³¹I. These doubly labeled cells were lysed, and H-2K^k and H-2D^k antigens were isolated by immunoprecipitation. Comparing the amounts at ¹²⁵I-labeled H-2K^k and H-2D^k antigens at times 0 and 8 h, the ratio of H-2K^k:H-2D^k fell from over 2:1 to nearly 1:1. This reflected the preferential loss of cell surface H-2K^k vs. H-2D molecules. However, the ratio of ¹³¹I-

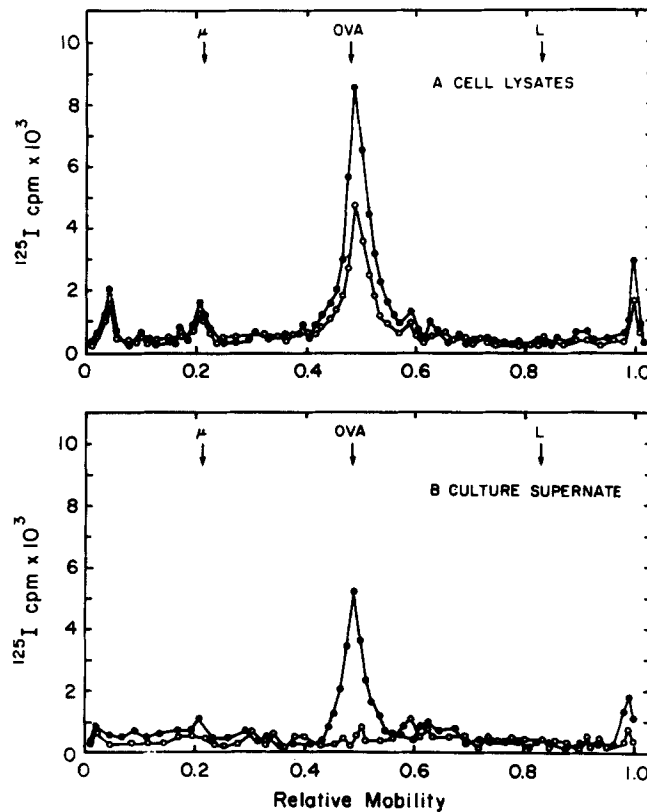


FIG. 2. SDS-PAGE of H-2 antigens isolated from CBA/J spleen cell lysates and culture supernates. Spleen cells were iodinated and incubated for 0–8 h. Cell lysates from the initial time point and culture supernates from the sample cultured for 8 h were dialyzed and apportioned for precipitation with anti-H-2K^k, anti-H-2D^k, and NMS. The *S. aureus* complexes were washed in 0.05% Triton X-100 until maximum specificity was achieved, and the bound immune complexes were solubilized and analyzed by SDS-PAGE under reducing conditions. ●—●, H-2K; ○—○, H-2D. (A) Electrophoretograms from 0-h cell lysates; (B) electrophoretogram from culture supernates. 11% acrylamide 0.25% bis-acrylamide; 80 1.5-mm slices per gel. NMS electrophoretograms were indistinguishable from background.

labeled H-2K^k vs. H-2D^k antigens at 8 h was greater than 2:1, indicating that over the incubation period, the total composition of the cell surface H-2 antigen population had remained stable. Thus, preferentially lost H-2K^k molecules must have been replaced at the cell surface during the culture period and therefore the selective H-2K^k shedding in CBA/J splenocytes represents the catabolic phase of net cell surface H-2K^k turnover.

Metabolism of the Cellular H-2 Antigen Pool is Similar to That of Cell Surface H-2 Molecules (Figs. 4 and 5). Biosynthetic labeling with [³H]leucine was used to compare the turnover of H-2K^k and H-2D^k molecules in the total cell pool, thus measuring intracellular as well as cell surface molecules. Single-cell suspensions of CBA/J splenocytes were cultured in leucine-free RPMI-1640 supplemented with [³H]leucine for 0–30 h. Portions were periodically removed, separated into cell and supernatant fractions, and analyzed by immunoprecipitation for the presence of leucine-labeled H-2K^k and H-2D^k antigens.

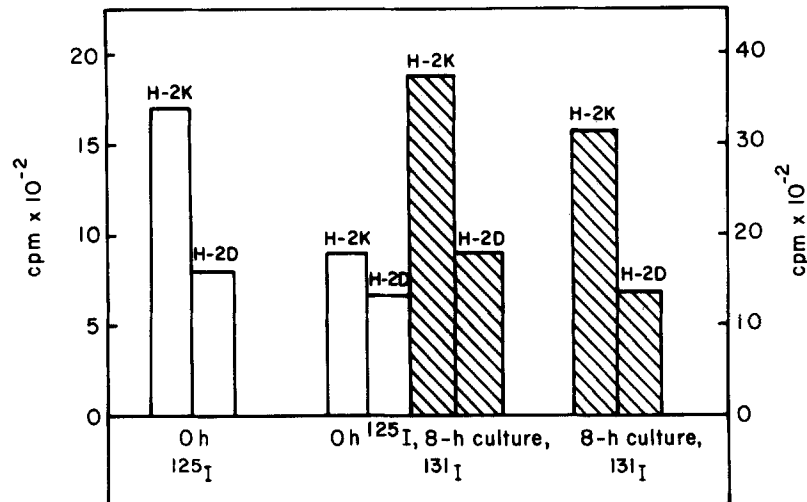


FIG. 3. Relabeling demonstrates that H-2K^k shedding from CBA/J spleen cells reflects net membrane antigen turnover. H-2K and H-2D antigen activities were measured in three parallel samples. In the first, ¹²⁵I-labeled (□) spleen cells were lysed at time 0, and specific H-2K and H-2D activities were determined. In the second, ¹²⁵I-labeled cells were cultured for 8 h, then centrifuged, washed, and relabeled with ¹³¹I (▨) (0-h ¹²⁵I). These double-labeled cells were then lysed, and specific ¹²⁵I and ¹³¹I H-2K and H-2D activities were simultaneously measured by immunoprecipitation (0 h ¹²⁵I, 8-h culture, ¹³¹I). In the third sample, unlabeled cells were cultured for 8 h, then labeled with ¹³¹I, and the specific activities of H-2K and H-2D in the cell lysates were assayed (8-h culture, ¹³¹I).

Radiolabeled H-2K^k and H-2D^k antigens were detected in cell lysates within 1 h of culture initiation. Leucine incorporation rose at a linear rate for both proteins over the next 11 h. After 12 h, however, net incorporation into H-2K^k molecules began to plateau whereas H-2D^k leucine levels continued to increase through over 20 h (Fig. 4). This more rapid plateau in H-2K^k leucine incorporation reflected the faster turnover of the whole cell H-2K^k pool because the plateau is observed when specific synthesis is precisely balanced by specific loss of synthesized H-2 molecules. The half-life for the whole cell H-2K^k pool, as determined by the time at which half-maximal leucine incorporation occurs (19), was approximately 8 h. No precise $t_{1/2}$ for cell H-2D^k turnover could be determined because a plateau in H-2D^k leucine incorporation was not observed, but the true half-life must be somewhat greater than 15 h.

Leucine-labeled H-2K^k molecules were first detected in culture supernates after 4 h of culture initiation, and accumulated thereafter at a gradually diminishing rate. Summation of cell plus supernatant H-2K^k leucine incorporation demonstrated that the rate of total net H-2K^k leucine incorporation was likewise nonlinear and decreasing with time. No leucine-labeled H-2D^k antigens could be isolated from culture supernates at any time during the incubation.

To determine whether the rapid shedding of cell surface H-2K^k molecules seen by cell surface radioiodination could be demonstrated by biosynthetic labeling, pulse-chase experiments were performed. Single cell suspensions were cultured in the presence of [³H]leucine for 4 h, then washed, and resuspended in complete RPMI-1640 medium for the remainder of the incubation. By this technique the fate of those H-2 molecules labeled during the 4-h isotope pulse could be determined. Pulse-labeled

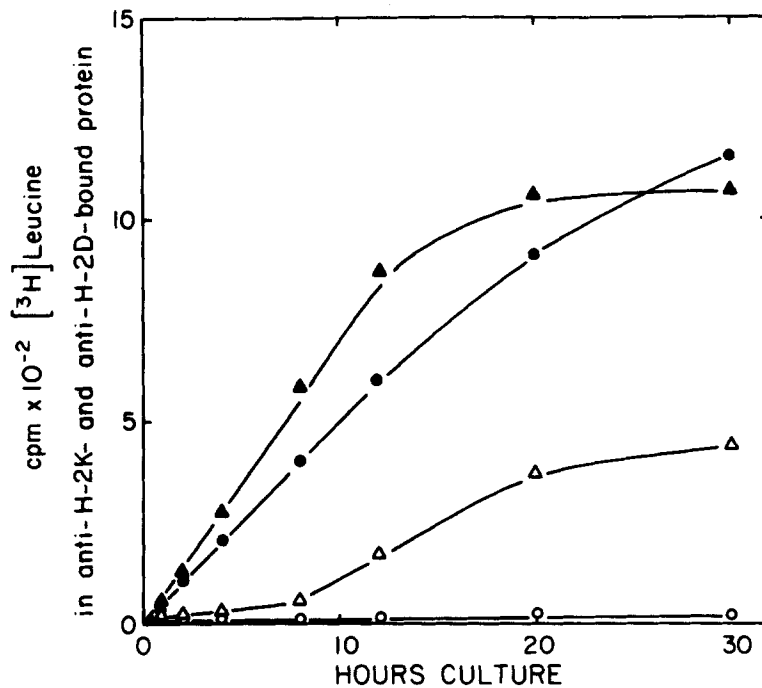


FIG. 4. Rapid turnover and release of H-2K antigens by CBA/J spleen cells demonstrated by continuous [^3H]leucine labeling. 5×10^6 CBA/J spleen cells were cultured for 0–30 h in the presence of [^3H]leucine. Portions were periodically withdrawn, separated into cell and supernatant fractions, and precipitated with anti-H-2K^k, anti-H-2D^k, and NMS. ▲—▲, H-2K^k cell; ●—●, H-2D^k cell; △—△, H-2K^k supernate; ○—○, H-2D^d supernate. Each point indicates experimental minus control counts per minute; the data shown are a representative example of one of three similar experiments.

H-2K^k antigens, which showed maximal [^3H]leucine incorporation at 4 h, declined rapidly during the chase period, with an initial $t_{1/2}$ of approximately 8 h; during this time the radiolabeled H-2K^k molecules appeared in the culture supernate (Fig. 5). Thus the pulse-chase experiments confirmed the rapid release of H-2K^k molecules, and overall the metabolism of the whole-cell H-2 pool closely resembles that of the cell surface H-2 molecules.

Analysis of H-2-congenic Strains Shows That the Relative Kinetics of H-2K and H-2D Antigen Shedding Is Controlled by the MHC (Table I). The relative shedding rates of H-2K and H-2D molecules were measured in several H-2-congenic mouse strains to determine whether the selective loss of H-2K molecules was a consistent finding. The results (Table I) demonstrated that a strict H-2K vs. H-2D preference was not always the case, and the patterns seen indicated strict genetic control of shedding rates linked to the MHC. Shedding rates in other H-2^k haplotype strains (C3H and B10.BR) were identical with those measured in CBA animals (H-2K^k $t_{1/2}$ = 8–9 h, H-2D^k = 30–35 h). Strains carrying the H-2^s haplotype (SJL, B10.S), however, shed H-2K^s and H-2D^s antigens with $t_{1/2}$ = 9–10 h. Similarly, both H-2K^b and H-2D^b were lost from cell surfaces of H-2^b spleen cells (B10, C57BL/6) with $t_{1/2}$ = 12 h. The preferential release of H-2K vs. H-2D antigens seen in the H-2^k haplotype was reversed in H-2^d mice (B10.D2, DBA/2), where H-2K^d $t_{1/2}$ = 40 h and H-2D^k $t_{1/2}$ = 10 h. Radiolabeled H-

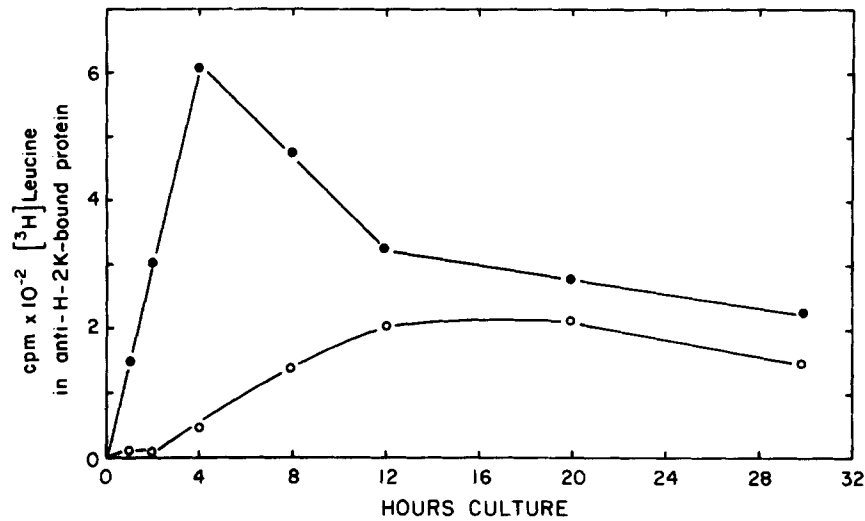


FIG. 5. Rapid loss of H-2K antigens from CBA/J spleen cells confirmed by pulse chase [³H]leucine administration. Spleen cells were cultured in the presence of [³H]leucine for 4 h, then washed, resuspended, and recultured in leucine-rich RPMI medium without isotope for 0-26 h. Samples were withdrawn at various time points, separated into cell and supernatant fractions, and portioned for immunoprecipitation with anti-H-2K* and NMS. ●—●, cell lysates H-2K antigens; ○—○, culture supernatant H-2K antigens. Each point indicates experimental minus control counts per minute; the data shown are a representative example of one of three similar experiments.

TABLE I
Turnover Rates of H-2K and H-2D Antigens are Influenced by Genes Mapping in the MHC

Mouse strain	H-2 haplo-type	H-2 regions*										Initial half-lives‡	
		K	I-A	I-B	I-J	I-E	I-C	S	G	D	H-2K	H-2D	
CBA/J	k	k	k	k	k	k	k	k	k	k	8	30	
C3H	k	k	k	k	k	k	k	k	k	k	9	35	
B10.BR	k	k	k	k	k	k	k	k	k	k	8	30	
B10.D2	d	d	d	d	d	d	d	d	d	d	40	10	
DBA/2	d	d	d	d	d	d	d	d	d	d	35	10	
SJL	s	s	s	s	s	s	s	s	s	s	10	10	
B10.S	s	s	s	s	s	s	s	s	s	s	10	9	
B10	b	b	b	b	b	b	b	b	b	b	12	13	
C57BL/6	b	b	b	b	b	b	b	b	b	b	12	12	

* See reference 24.

‡ Spleen cells were iodinated and incubated for 20 h. Portions were periodically removed from cultures, lysed, and analyzed by immunoprecipitation for the presence of radiolabeled H-2K and H-2D antigens. Initial half-lives were determined by measuring the slope of the initial, most rapid phase of H-2 antigen decline. Values shown are the mean of three measurements.

H-2D antigens were, in fact, recovered from B10.D2 culture supernates (data not shown). Thus the turnover rates of H-2K and H-2D antigens were very similar in strains with the same H-2 haplotype on different backgrounds, but varied considerably between different H-2 haplotypes on the same genetic background.

There are several possible explanations for the observed H-2 control of H-2K and H-2D antigen turnover. First, products of different alleles at one locus may be

differentially shed (perhaps caused by variation in the primary sequence of these molecules). Second, the turnover of products of different alleles at one locus may be regulated by another H-2-linked locus. Third, both alternatives may be correct. In order to explore the second possibility, we examined several H-2-recombinant strains.

The k Allele at a Locus Mapping to the Left of the I-A Subregion Depresses the Rate at which H-2D^d Antigens Are Shed. Study of H-2 Recombinants (Tables II and III). Analysis of recombinant strains B10.A and A/Sn revealed that the shedding rate of the H-2D^d antigen is depressed by the *k* allele at a locus mapping in the K end (i.e., toward the centromeric end of the I-C subregion) of the H-2 gene complex. For example, H-2D^d molecules are shed rapidly from strain B10.D2 and DBA/2 splenocytes ($t_{1/2} = 10$ h) but slowly from strain B10.A or A/Sn splenocytes ($t_{1/2} = 30$ h) (Table II). Thus, a *d*-derived locus mapping to the left of the I-C subregion results in rapid H-2D^d antigen turnover, whereas a *k*-derived locus mapping in the same interval depresses H-2D^d antigen turnover.

Study of recombinant strains B10.A(4R) (K^k-D^b), B10.S(7R) (K^s-D^d), and B10.A(5R) (K^b-D^d) revealed no obvious regulation of H-2K or H-2D antigen turnover by another locus. In addition, results with strain B10.A(4R) reveal that H-2D^b antigen turnover ($t_{1/2} = 16$ h) is not depressed by the *k* allele at the locus that depresses H-2D^d antigen turnover. The *k* allele at this regulatory locus, therefore, does not nonspecifically influence H-2D antigen turnover.

Data in Table III localize the *k*-derived regulatory locus to the left of the I-A

TABLE II
H-2D^d Antigen Turnover is Depressed in Strains Bearing a k-derived Locus

Mouse strain	H-2 haplo-type	H-2 regions*										Initial half-lives‡	
		K	I-A	I-B	I-J	I-E	I-C	S	G	D	H-2K	H-2D	
B10.D2	d	d	d	d	d	d	d	d	d	d	d	40	10
B10.A	a	k	k	k	k	k	d	d	d	d	d	8	30
A/J	a	k	k	k	k	k	d	d	d	d	d	9	30
B10.A(4R)	h4	k	k	b	b	b	b	b	b	b	b	8	16
B10.S(7R)	t2	s	s	s	s	s	s	s	s	s	d	10	10
B10.A(5R)	i5	b	b	b	b	d	d	d	d	d	d	13	9

* See reference 24.

‡ Half-lives were determined as described in Table I.

TABLE III
A k-derived Locus Mapping to the Left of the I-A Subregion Depresses H-2D^d Antigen Turnover

Mouse strain	H-2 haplo-type	H-2 regions*										Initial half-lives‡	
		K	I-A	I-B	I-J	I-E	I-C	S	G	D	H-2K	H-2D	
B10.D2	d	d	d	d	d	d	d	d	d	d	d	40	10
A.TH	t2	s	s	s	s	s	s	s	s	s	d	10	10
A.TL	t1	s	k	k	k	k	k	d	d	d	d	11	10
A.AL	a1	k	k	k	k	k	k	d	d	d	d	8	40

* See reference 24.

‡ Half-lives were determined as in Table I.

subregion. For example, H-2D^d antigen loss was very slow ($t_{1/2} = 40$ h) with recombinant strain A.AL (K^kI^kD^d) spleen cells. In contrast, H-2D^d antigen loss was rapid ($t_{1/2} = 10$ h) with recombinant strain A.TL (K^sI^kD^d) spleen cells. No apparent influence of I region loci on H-2D^d antigen turnover was observed (strain A.TH (K^sI^sD^d), $t_{1/2} = 10$ h). Thus, the *k* allele at a locus mapping to the left (toward the centromere) of the I-A subregion depresses H-2D^d antigen release.

Discussion

The present study demonstrates that murine spleen cells shed one or both of their cell surface H-2 antigens. Moreover, the released H-2 antigens are stoichiometrically replaced at the cell surface, resulting in a continual, rapid regeneration of newly inserted H-2 molecules. This observation and the fact that the decrease in cell viability and number during culture was minimal indicate that the loss of H-2 antigens was not caused by dying cells. Moreover, in studies to be published elsewhere we demonstrate that the shedding of H-2 antigens (as well as I-A, membrane IgM and IgD) is an active process of viable cells involving cytoskeletal elements. (S. G. Emerson and R. E. Cone. Manuscript submitted for publication).

SDS-PAGE analysis of shed H-2 antigens demonstrated that no other membrane proteins were coprecipitated with the H-2 antigens. Thus, the shed antigens are not associated with a large random fragment of membrane. However, ultracentrifugation, gel filtration, and biosynthetic labeling with lipid precursors indicate that the H-2 antigens are shed in association with membrane lipids in particles of 10^5 – 2×10^6 d (S. G. Emerson and R. E. Cone. Manuscript submitted for publication). H-2 antigens released by the cells do not remain stable. Thus, while the amount of radiolabeled H-2K^k in CBA/J culture supernates increases during the beginning of the incubation period, measurements after 8 h indicate subsequent decline in supernatant H-2K^k levels. This decrease, detected by both ¹²⁵I-surface label and pulse-chase [³H]leucine label (Figs. 1B and 5), could be the result of proteolysis, denaturation, or absorption of the shed product by other lymphoid cells. Experiments now in progress indicate that absorption of shed H-2 antigens does indeed occur in these cultures.

Whereas all H-2^k strains tested, regardless of genetic background, preferentially shed H-2K vs. H-2D antigens, other haplotypes show different patterns (Table I). H-2K and H-2D antigens are shed from H-2^s cells at identical rates ($t_{1/2} = 10$ h); likewise both H-2K and H-2D antigens are lost from H-2^b cells with initial $t_{1/2} = 12$ h. H-2^d splenocytes show selective shedding opposite to the H-2^k mice: H-2K^d molecules are lost very slowly ($t_{1/2} = 40$ h), whereas H-2D^d antigens are shed rapidly ($t_{1/2} = 10$ h). Examination of intra-H-2-recombinant strains demonstrated that the turnover rate of an individual H-2 molecule can be regulated by another locus. Thus whereas H-2D^d shedding on B10.D2 and DBA/2 cells is rapid ($t_{1/2} = 10$ h), H-2D^d loss from B10.A and A splenocytes is very slow ($t_{1/2} = 30$ h). These results suggest that the loss kinetics of H-2D^d antigens are regulated by MHC genes mapping to the left of the I-C subregion. Because H-2D^d antigens are lost rapidly from A.TL (K^sI^kD^d) cells and slowly from A.AL (K^kI^kD^d) cells, this regulatory locus maps to the left of the I-A subregion. Whether this regulatory locus maps in the K region or to the left of the K region (toward the centromere) remains to be determined. Recently, a new subregion, I-N, which maps between the K region and I-A subregion, has been reported (20). Because strain TL carries the I-N^s subregion, and strain A.AL carries the I-N^k

subregion, our regulatory locus could conceivably map in this segment of chromosome. In addition, we would emphasize that the regulation of H-2D antigen turnover by a locus mapping to the left of the I-A subregion has been demonstrated in only one combination. Whether this regulation holds for other combinations is currently being explored.

These variations in turnover and shedding rates, whatever the precise genetic and molecular mechanism, show a marked correlation with reported genetic variations in quantitative cytotoxic responses to chemically modified and virus-infected syngeneic cells (7-11, 21, 22). In studies of TNP-modified syngeneic cells, strong anti-TNP cytotoxic responses were generated in association with H-2K^b and H-2K^k; strong anti-TNP responses were generated in association with H-2D^d only in strains that lacked the K^d region. TNP-H-2K^d was always a poor stimulator of cytotoxicity (7, 8, 21). Moreover, the regulatory effect of *k* alleles on TNP-H-2D^d recognition was mapped to the K region (8), utilizing the same strains examined in the present study. Similarly, responses to vaccinia, Sendai, and SV-40 were preferentially observed in the presence of H-2K^k and H-2K^b, and with H-2D^d in strains that do not carry the K^k region (9, 11). H-2D^k antigens were poor costimulators for anti-virus cytotoxic responses for these viruses, as well as for influenza (9-11). In addition, resistance to radiation-induced leukemia virus infection has been shown to correlate with increased expression and synthesis of H-2D^d antigens in thymocytes (22).

Taken together, these quantitative cytotoxic restrictions and the present preferential shedding data suggest that rapid turnover of H-2 antigens plays a key role in T cell recognition of H-2-associated foreign antigens. Rapidly shed H-2 molecules, whether coded by loci in the K or D regions, appear to be preferred vehicles for stimulation of T cell-cytotoxic response. Weak cytotoxic responses to chemically modified or virus-infected syngeneic cells of a given haplotype could thus be results of the stimulating cell and not necessarily of the capacity of T lymphocytes to respond to these antigens. It should be stressed that in the present study we have examined constitutive H-2 turnover. The possibility that chemical modification or virus infection alters normal turnover rates (22) needs to be explored further.

The mechanisms by which rapid turnover might allow for the generation of specific cytotoxic T cells is not known. However, shedding of TNP-modified H-2 antigens, or of H-2 antigens in association with viral proteins, would effectively increase the functional stimulation radius of the modified or infected cell. Instead of the stimulating antigen-H-2 molecule unit being limited to the cell surface, rapid shedding would seed the milieu with antigenic moieties, thus increasing the likelihood of stimulating a nearby precursor of a specifically cytotoxic T cell. An alternative explanation, at least for the virally infected stimulator cells, might be that rapid turnover allows for the expression of many more molecules of viral proteins in association with H-2 antigens per unit time. Thus, because shedding is accompanied by stoichiometric replacement at the cell surface, the number of antigens per unit time appearing on stimulator cell membranes would be greatly increased by rapid H-2 turnover. This explanation could not hold for TNP-modified autologous cells, however, as the total amount of TNP-self available for stimulation is always limited by the initial cell surface TNP labeling, and could not be increased thereafter by H-2 turnover.

These variations in H-2 antigen shedding rates may also explain the discrepancies among previous reports of H-2 turnover (15, 23, 24). Whereas Forman and Vitetta

(14) used a specific anti-H-2K^k antiserum and detected rapid H-2 loss, the earlier studies, employing sera which recognized both H-2K^k and H-2D^d molecules, found slower turnover (23, 24). If these poly-specific sera had strong reactivities for the H-2D^d molecules, but weak anti-H-2K^k, very slow turnover might well be observed. The present investigations clarify this apparent conflict by demonstrating that some, but not all, H-2 antigens undergo rapid shedding and turnover. Moreover, this variation in turnover rates suggests that this shedding process may be instrumental in the presentation of foreign membrane-bound antigens to the immune system.

Summary

Lactoperoxidase-catalyzed cell surface radioiodination and incorporation of [³H]-leucine were employed to radiolabel H-2K and H-2D antigens of murine spleen cells. The fate of H-2 antigens was monitored by in vitro culture of labeled cells and isolation of labeled antigens from detergent lysates of the cells and culture supernates obtained at different times during culture. H-2K^k antigens were found to be rapidly turned over and shed by CBA/J cells, whereas the turnover of H-2D^k antigens was extremely slow. Analysis of the membrane residence times of surface-labeled H-2K and H-2D antigens on spleen cells from various H-2-congenic and -recombinant strains demonstrated variations in the shedding rates of H-2K and H-2D antigens, which were controlled by genes mapping in the major histocompatibility complex. These variations show a striking correlation with published, genetically controlled quantitative variations in the cytotoxic response of T lymphocytes to chemically modified or virus-infected syngeneic cells.

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References

1. Zinkernagel, R. M., and P. C. Doherty. 1975. H-2 compatibility requirement for T cell mediated lysis of target cells infected with lymphocytic choriomeningitis virus. Different cytotoxic T cell specificities are associated with structures coded for in H-2K or H-2D. *J. Exp. Med.* **141**:1427.
2. Gardner, I. D., N. A. Bower, and R. V. Blanden. 1975. Cell-mediated cytotoxicity against ectromelia virus-infected target cells. III. Role of the H-2 gene complex. *Eur. J. Immunol.* **5**: 122.
3. Bevan, M. J. 1975. Interaction antigens detected by cytotoxic T cells with the major histocompatibility complex as modifier. *Nature (Lond.)* **256**:419.
4. Gordon, R. D., E. Simpson, and L. E. Samelson. 1975. In vitro cell-mediated immune responses to the male specific (H-Y) antigen in mice. *J. Exp. Med.* **142**:1108.
5. Shearer, G. M., T. G. Rehn, and C. A. Garbarine. 1975. Cell-mediated lympholysis of trinitrophenyl-modified autologous. Effector cell specificity to modified cell surface components controlled by the H-2K and H-2D serological regions of the murine major histocompatibility complex. *J. Exp. Med.* **141**:1348.
6. Forman, J. 1975. On the role of the H-2 histocompatibility complex in determining the

- specificity of cytotoxic effector cells sensitized against syngeneic trinitrophenyl-modified targets. *J. Exp. Med.* **142**:403.
7. Schmitt-Verhulst, A. M., and G. M. Shearer. 1975. Bifunctional major histocompatibility-linked genetic regulation of cell-mediated lympholysis to trinitrophenyl-modified autologous lymphocytes. *J. Exp. Med.* **142**:914.
 8. Schmitt-Verhulst, A. M., and G. M. Shearer. 1976. Multiple H-2-linked immune response gene control of H-2D-associated T-cell-mediated lympholysis to trinitrophenyl-modified autologous cells. Ir-like genes mapping to the left of I-A and within the I region. *J. Exp. Med.* **144**:1701.
 9. Zinkernagel, R. M., A. Althage, S. Cooper, G. Kreeb, C. A. Klein, B. Sefton, L. Flaherty, J. Stimpfling, D. Schreffler, and J. Klein. 1978. Ir-genes in H-2 regulated generation of anti-viral cytotoxic T cells. *J. Exp. Med.* **148**:592.
 10. Doherty, P. C., H. E. Biddison, J. R. Bennink, and B. B. Knowles. 1978. Cytotoxic T-cell responses in mice infected with influenza and voicinia viruses vary in magnitude with H-2 genotype. *J. Exp. Med.* **148**:534.
 11. Pfizenmaier, K., G. Trinchieri, D. Solter, and B. B. Knowles. 1978. Mapping of H-2 genes associated with T cell-mediated cytotoxic responses to SV-40-tumour-associated specific antigens. *Nature (Lond.)*. **274**:691.
 12. Simpson, E., and R. D. Gordon. 1972. Responsiveness to H-Y antigen Ir gene complementation and target cell specificity. *Immunol. Rev.* **35**:59.
 13. Emerson, S. G., and R. E. Cone. 1979. Turnover and shedding of Ia antigens by murine spleen cells in culture. *J. Immunol.* **122**:892.
 14. Forman, J., and E. S. Vitetta. 1978. Relationship between trinitrophenol and H-2 antigens on trinitrophenyl-modified spleen cells. IV. Correlation between the loss of cell surface trinitrophenyl-H-2 molecules and functional activity of derivatized cells in an anti-TNP CML assay. *J. Immunol.* **121**:1002.
 15. Forsgren, A., and J. Sjoquist. 1967. Protein A from *Staphylococcus aureus*. III. Reaction with rabbit-globulin. *J. Immunol.* **105**:1116.
 16. Kronvoll, G., and D. Frommel. 1970. Definitions of staphylococcal protein A reactivity for human immunoglobulin G fragments. *Immunochemistry*. **7**:124.
 17. Kessler, S. 1976. Cell membrane antigen isolation with the staphylococcal A antibody absorbent. *J. Immunol.* **117**:1482.
 18. Cone, R. E., and W. Brown. 1976. Isolation of membrane-associated immunoglobulins from T lymphocytes by non-ionic detergents. *Immunochemistry*. **13**:571.
 19. Schimke, R. T. 1977. Turnover of membrane proteins of animal cells. *Annu. Rev. Biochem.* **46**:203.
 20. Hayes, C. E., and F. H. Bach. 1980. I-N. A newly described H-2I subregion between K and I-A. *J. Exp. Med.* **157**:481.
 21. Levy, R. B., and G. M. Shearer. 1979. Regulation of T-cell mediated lympholysis by the murine major histocompatibility complex. I. Preferential in vitro responses to trinitrophenyl-modified parental and F₁ hybrid mouse strains. *J. Exp. Med.* **149**:1379.
 22. Meruelo, D., S. H. Nimelstein, P. Jones, M. Lieberman, and H. O. McDevitt. 1978. Increased synthesis and expression of H-2 antigens on thymocytes as a result of radiation leukemia virus infection: a possible mechanism for H-2-linked control of virus-induced neoplasia. *J. Exp. Med.* **147**:470.
 23. Wernet, D., E. S. Vitetta, J. W. Uhr, and E. A. Boyse. 1973. Synthesis, intracellular distribution, and secretion of immunoglobulin and H-2 antigen in murine splenocytes. *J. Exp. Med.* **138**:847.
 24. Vitetta, E. S., and J. W. Uhr. 1975. Synthesis of surface H-2 alloantigens in murine splenocytes. *J. Immunol.* **115**:374.