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Alloreactive cytolytic T-cell clones preferentially recognize conformational determinants on histocompatibility antigens: Analysis with genetically engineered hybrid antigens

(deletion subcloning/exon shuffling/allodeterminants/monoclonal antibodies)

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ABSTRACT **Hybrid genes were constructed for the localization of allodeterminants on murine class I antigens recognized by antibodies and cytolytic T lymphocytes. By using deletion subclones of the *H-2K*^{d} and *H-2K*^{k} genes, homologous regions were exchanged between the two alleles. The altered genes were introduced and expressed in mouse fibroblast and fibrosarcoma cells. Cells expressing hybrid antigens were analyzed with 29 monoclonal anti-H-2K^d and anti-H-2K^k antibodies and with 150 short-term alloreactive cytolytic T-cell clones. When only the first or only the second amino-terminal domain was exchanged, most T cells and 60% of the antibodies lost their reactivity to the H-2K antigen. No T-cell clone was directed against the third extracellular domain, whereas three antibodies could bind to this domain. This implies that nearly all determinants essential for a cytolytic T-cell response or for antibody binding lie on the two external domains and are conformational structures generated by the interaction of these two domains.**

Class I genes of the murine major histocompatibility complex (MHC) encode the serologically defined transplantation antigens H-2K, H-2D, and H-2L. A large percentage of T lymphocytes seems to be obsessed with the recognition of these class I histocompatibility antigens. For example, graft rejection is based on the recognition of allogeneic transplantation antigens by T lymphocytes. In addition, the elimination of virally infected or neoplastically transformed cells by T lymphocytes always requires recognition of self-determinants contained on class I antigens together with the viral or tumor antigens (1, 2).

The exon–intron organization of class I genes corresponds to the discrete regions of the protein (3). The first exon encodes the signal sequence; exons 2, 3, and 4 encode the three extracellular domains α1, α2, and α3, and exons 5–8 encode the transmembrane and cytoplasmic segments. To investigate which region of the class I antigens is of importance for cellular interactions in the immune system, homologous exons between different class I genes were exchanged in previous studies, and the resulting hybrid genes were expressed in fibroblast lines. The results suggested that the α1 and α2 domains are important for recognition by cytotoxic T lymphocytes (CTL) (4). Conflicting results were obtained as to whether or not α1 and α2 can be recognized independently of one another. Anti-H-2D^d CTL were able to recognize efficiently an H-2D^d/L^d hybrid with only the α1 domain of H-2D^d (5). In contrast, exchange of α1 between H-2K^b/D^b or H-2K^k/K^d led to abrogation of CTL recognition (6, 7). All these studies have been performed with CTL generated in bulk cultures and few long-term CTL clones. In addition, the effect of an exchanged α2 domain has not been studied previously.

In the present report we describe a series of hybrid genes between *H-2K*^{d} and *H-2K*^{k} genes, including those with only exchanged α2 domains. Cells expressing these hybrid antigens were investigated with CTL from bulk cultures, with 150 short-term CTL clones, and with 29 monoclonal anti-H-2K^k and anti-H-2K^d antibodies. Our results show that most but not all T-cell clones and antibodies are strongly influenced in their reactivity if the α1 and α2 domains are of different alleles. These findings suggest that the majority of allodeterminants are conformational structures generated by interaction α1 and α2 domains.

MATERIALS AND METHODS

Construction of Hybrid *H-2K* Genes. The *H-2K*^{d} gene and the *H-2K*^{k} gene have been characterized extensively (8, 9). The construction of hybrid genes, plasmids pC31, pC32, and pC33, from the *H-2K*^{d} and *H-2K*^{k} genes has been described in detail recently (7). We used the same deletion subcloning method (7) to construct more hybrid genes, plasmids pC23, pC24, and pC25 (Fig. 1*A*).

To generate *H-2K* hybrid genes in which only exon III coding for the α2 domain is exchanged, we combined the deletion subcloning method with the use of endogenous restriction sites present in the genes (Fig. 1 *B* and *C*). The *H-2K*^{k} and the *H-2K*^{d} genes each have an *Xba* I site 400 nucleotides upstream of exon I and an additional *Xba* I site in intron III. Digestion of these genes or of the hybrid genes with *Xba* I endonuclease therefore creates DNA fragments containing exons I, II, and III. These *Xba* I fragments of the plasmids pC31 and pC25 were ligated into the *Xba* I site of subclones of *H-2K*^{d} or *H-2K*^{k} in which the first three exons have been deleted (Fig. 1 *B* and *C*). In this way the hybrid genes designated plasmids pC26 and pC35 were obtained.

Cell Culture and DNA Transfection of Cells. Two cell lines were used in our transfection experiments: the fibroblast cell line 1T 22-6 (7), which is of *H-2*^{q} origin, and the methylcholanthrene-induced fibrosarcoma line IC9, which is of (C57BL/6 × C3H)F$_1$ origin (supplied by S. Segal). The latter cell line expresses only the H-2D^b MHC antigen (10). Cells were grown in Dulbecco's minimal essential medium containing 10% fetal calf serum. *H-2*-specific DNA was cotransfected with the neophosphotransferase gene using the calcium-phosphate method as described (7).

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Abbreviations: CTL, cytotoxic T lymphocyte; FACS, fluorescence activated cell sorter; MLC, mixed lymphocyte culture; MHC, major histocompatibility complex.

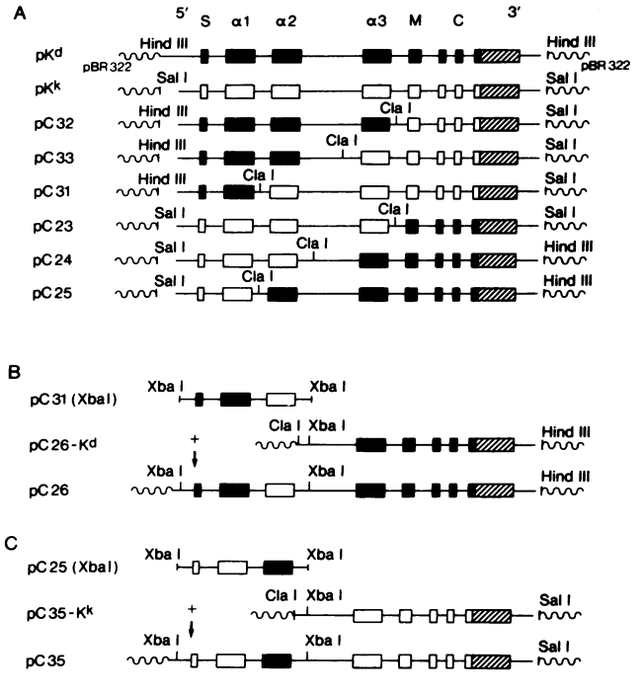

FIG. 1. Plasmids with hybrid genes of the *H-2K*^{d} and *H-2K*^{k} alleles. (*A*) Hybrid genes constructed by joining subclones of partially deleted parental *H-2K* genes. The *Cla* I site in all plasmids indicates the site of recombination. The exon–intron organization of the two parental *H-2K* genes is shown above the top line. Abbreviations indicate the protein regions for which the exons are coding: S, signal sequence; α1, α2, and α3, the three extracellular domains; M, the transmembrane portion; C, the cytoplasmic part. The hatched boxes indicate the 3′ untranslated regions, filled boxes denote exons of *H-2K*^{d} origin, and open boxes denote exons of *H-2K*^{k} origin. (*B* and *C*) Exchange of exon III by combining the deletion subcloning method with the use of endogenous restriction endonuclease *Xba* I sites. See *Materials and Methods* for further details.

Cytofluorography. The properties of monoclonal antibodies used for the analysis of the transfected cells by the fluorescence activated cell sorter (FACS) are summarized in Table 1. FACS analyses were carried out as described (7).

Mixed Lymphocyte Cultures (MLC) and Limiting Dilution System for the Generation of Alloreactive CTL Clones. Alloreactive CTLs were generated in MLC by stimulation of 10^7 B10 spleen cells with 10^7 irradiated B10.A(4R) (anti-H-2K^k CTLs) or with 10^7 irradiated B10.GD (anti-H-2K^d CTLs) spleen cells as described (11). These bulk cultures were tested for their specificity 5 days later and then restimulated under limiting dilution conditions as described earlier (11). The resulting CTL clones were tested for their specificity, and the positive ones were expanded by restimulation. The CTL clones were tested in duplicate 8–10 days later on four different target cell lines using standard chromium release assays (11).

RESULTS

Construction and Expression of *H-2K*^{d}/*K*^{k} Hybrid Genes. All plasmids with hybrid *H-2K*^{d}/*K*^{k} genes used in this study are listed in Fig. 1. We have recently described the construction of pC31, pC32, and pC33 (Fig. 1*A*). pC23, pC24, and pC25 were made in the same way (Fig. 1*A*). For the construction of pC26 and pC35, exon III, encoding the α2 domain, was replaced by exon III of the other allele. (See *Materials and Methods* for details).

All hybrid genes shown in Fig. 1 were transfected into 1T 22-6 fibroblasts, and the resulting cell lines were characterized by immunoprecipitation and subsequent analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (data not shown). By FACS analysis it was ascertained that approximately equal amounts of hybrid antigens were expressed on the cell surface of the various cell lines. Fig. 2 shows such an analysis with the cell line C26-27 as an example. Antibody 20-8-4S could detect C26 on the cell surface of C26-27 cells, but the binding was reduced in comparison to the binding of this antibody to cell line K^d-4 expressing the wild-type H-2K^d molecule (Fig. 2 *A* and *C*). The new monoclonal antibody K9-18 (unpublished data) is specific for the α3 domain of H-2K^d. In contrast to the antibody 20-8-4S, this antibody bound equally well to the cell lines C26-27 and K^d-4 (Fig. 2 *B* and *D*).

Alloreactive CTL Lysis of Target Cells Expressing Hybrid H-2K Antigens. The series of H-2K^d/K^k hybrid antigen-expressing cell lines allowed us to study whether determinants for alloreactive CTL are expressed on individual domains or are created by interaction between the α1, α2, and α3 domains. Anti-H-2K^d or anti-H-2K^k CTL from such primary bulk cultures could specifically lyse 1T 22-6 fibroblasts expressing the corresponding authentic H-2K molecule and cells expressing hybrid antigens with the three or two extracellular domains of the corresponding *H-2K* allele (Fig. 3 *A–C, G, H,* and *I*). The four cell lines (C31-47, C35-4, and C25-20 and C26-27) that expressed hybrid antigens with α1 and α2 domains of different alleles, could not be detectably lysed by anti-H-2K^k or anti-H-2K^d CTL populations (Fig. 3 *D, E, J,* and *K*). In contrast, all cell lines were lysed to about the same extent by anti-H-2^q (B10 anti-B10.G) CTL as is shown for the cell lines C33-2 and C31-47 (Fig. 3*F*) and C24-19 and C25-20 (Fig. 3*L*), implying that they are not resistant to lysis.

Since bulk cultures of alloreactive anti-H-2K^k and anti-H-2K^d CTL could not effectively lyse target cells expressing hybrid antigens with α1 or α2 domains exchanged, we examined the reaction pattern of T-cell clones. The use of

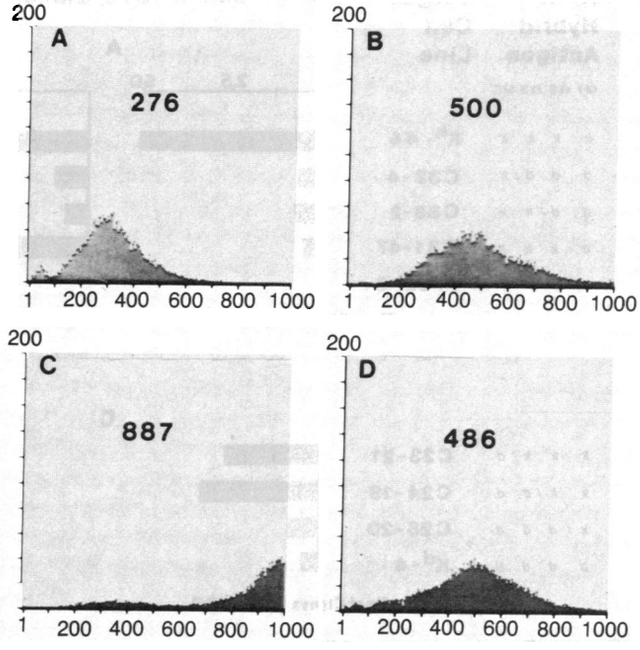

FIG. 2. Cell-surface expression of the hybrid antigen C26. Cell clones C26-23 and K^d-4 were allowed to react with the monoclonal anti-H-2K^d antibodies 20-8-4S (*A* and *C*) and K9-18 (*B* and *D*) and were stained with fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin serum. The fluorescence profiles were obtained by analyzing 3 × 10^4 cells in an Ortho 50H cytofluorograph. The numbers denote the mean values of fluorescence intensity.

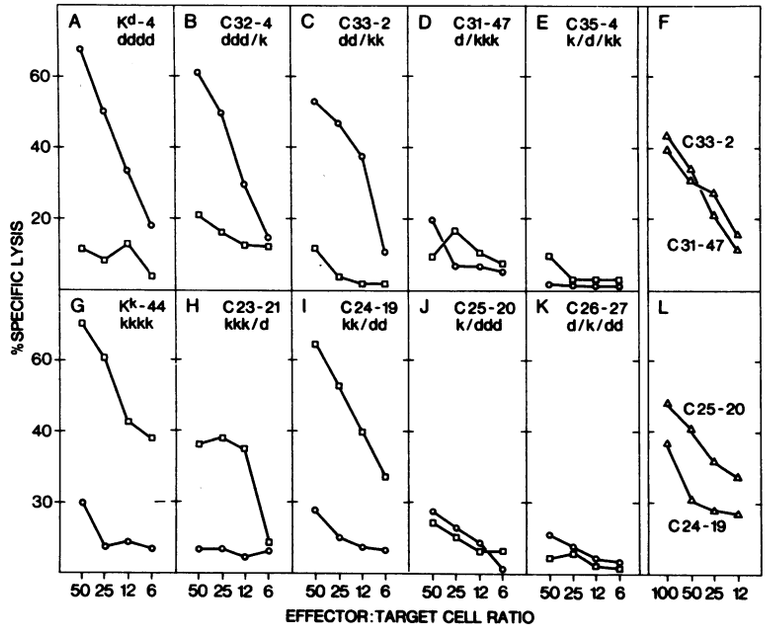

FIG. 3. Analysis of cell lines expressing H-2K hybrid antigens with bulk cultures of CTL. The transfected cell lines were labeled with ^{51}Cr and assayed for their ability to constitute targets for anti-H-2K^d CTLs (B10 anti B10.GD) (○) and anti-H-2K^k CTLs (B10 anti-B10.A(4R) (□) (*A–E* and *G–K*). In addition anti-H-2^q CTLs (B10 anti-B10.G) (△) were used (*F* and *L*). The percentages of lysis are mean values of triplicate wells analyzed. Identical results were obtained in at least three independent experiments. The spontaneous release of ^{51}Cr varied between 6% and 14%. The four small letters below the number of each cell line indicate the *H-2K* allele of the three external domains α1, α2, and α3 and the COOH-terminal part of the antigen.

limiting dilution systems allowed us to use a large number of individual CTL clones in contrast to the limited number of long-term CTL clones analyzed by other investigators. The reaction patterns obtained are shown in Fig. 4. Of 46 anti-H-2K^k T-cell clones, 45 could lyse 1T 22-6 fibroblasts transfected with the *H-2K^k* gene but had no effect on target

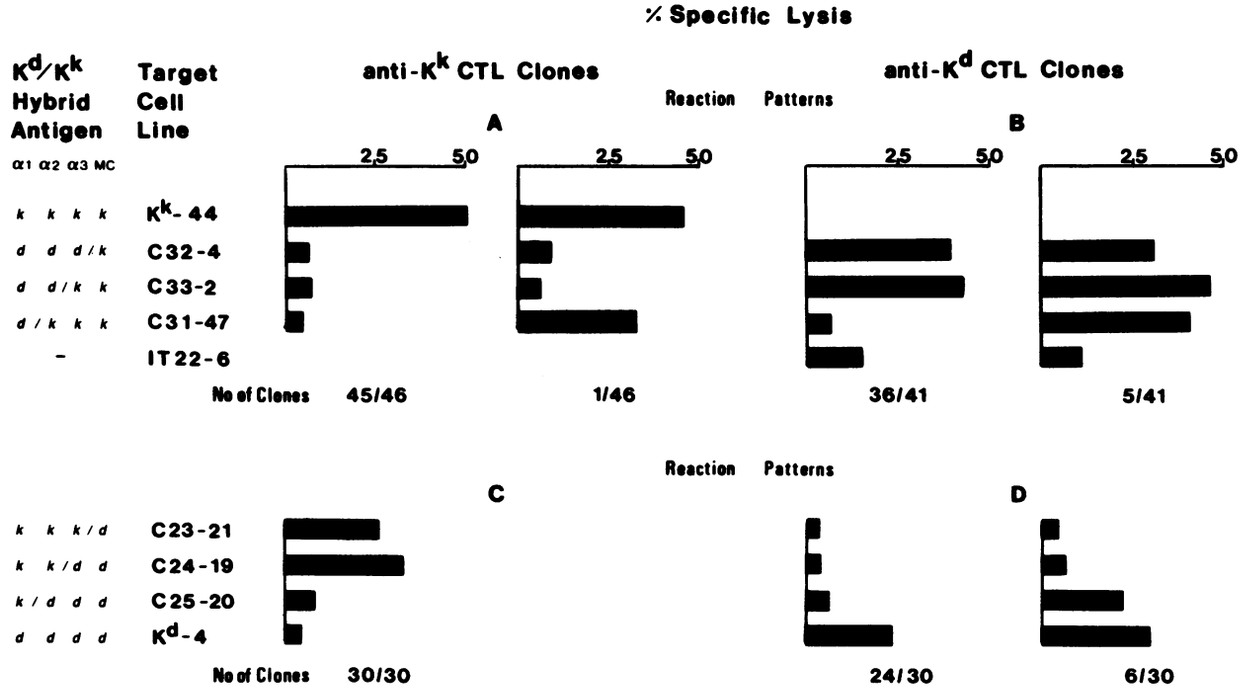

FIG. 4. Reaction patterns of CTL clones tested on cell lines expressing hybrid H-2K antigens. T-cell clones obtained from limiting dilutions were split into eight parts and tested on four target cell lines in duplicate. The effector-to-target ratio was 20:1. CTL clones were scored as responders when the ^{51}Cr release exceeded the mean spontaneous ^{51}Cr release by more than 3 standard deviations. The two reaction patterns of 46 anti-H-2K^k and 41 anti-H-2K^d CTL clones are given in *A* and *B*, respectively. The number of clones showing such a reaction pattern is given underneath each figure. Reaction patterns given in *C* and *D* represent the lysis of four other target cell lines by 30 CTL clones of each specificity. Since all 30 anti-H-2K^k CTL clones showed the same lysis characteristics, only one reaction pattern is given in *C*. The small letters (left in the figure) indicate the allele of the extracellular domains α1, α2, and α3 and the transmembrane and cytoplasmic portion (MC) of the hybrid antigen.

cells expressing the hybrid antigens C32, C33, and C31. Only 1 of 46 anti-H-2K^k T-cell clones could lyse cell line C31-47 as well as cell line K^k-44 (Fig. 4*A*). Similar results were obtained with anti-H-2K^d CTL clones. Only a minority (5 of 41) could lyse cell line C31-47, whereas the majority of clones (36 of 41) showed the reaction pattern known from CTL bulk cultures (Fig. 4*B*). Another series of 30 anti-H-2K^k and 30 anti-H-2K^d CTL clones were tested on the cell lines C23-21, C24-19, C25-20, and K^d-4 (Fig. 4 *C* and *D*). Again, either no anti-H-2K^k (0 of 30) or a small but significant percentage of anti-H-2K^d (6 of 30) CTL clones could lyse cell line C25-20 expressing the hybrid antigen with the α1 domain of H-2K^k and the α2 domain of H-2K^k, whereas all cell clones lysed target cells expressing antigens with the two outer domains of the same allele.

We conclude from these data that α1 and α2 of the H-2K antigen have to be of the same allele to allow a reaction of the vast majority of T cells. On the other hand CTL clones can be found with specificity for H-2K antigens with α1 and α2 of different alleles, if the number of tested clones is high enough.

Localization of Serological Determinants on H-2K^k and H-2K^d Antigens. We have used the H-2K^k/K^d hybrid antigens to examine the location of serological determinants and to investigate how these determinants are influenced by other regions of the H-2 molecule. Since most of the monoclonal anti-H-2K^k antibodies were crossreactive with H-2^q, we transfected IC9 fibrosarcoma cells expressing only the H-2D^b MHC antigen. The transfected cell lines were characterized by immunoprecipitation and FACS analysis. The antibodies K9-18 and S13-29 were found to react with the α3 domain of the H-2K^d antigen or the α3 domain of the H-2K^k antigen, respectively, and were not influenced in their binding capacity by the exchange of α1 or α2 between the alleles (Table 1). Therefore, we used these antibodies for FACS analysis of the transfected lines to select cell lines that expressed the hybrid antigens in equal amounts; 20 monoclonal anti-H-2K^k and 9 monoclonal anti-H-2K^d antibodies were characterized (Table 1). The α1 domain of the corresponding allele was sufficient to allow binding of 8 of 29 antibodies, but the binding efficiency was reduced in all cases (patterns A and D). Expression of antigens with the α1 and the α2 domains of the same allele was essential for the binding of 18 of 29 antibodies (patterns B and E). Only 3 of 29 antibodies reacted with a determinant on α3 but with the same fluorescence intensity as on the authentic H-2K antigen (patterns C and F). The monoclonal antibodies S13.29 and S13.11 were crossreactive between H-2K^k and H-2K^d, but cells expressing hybrid antigens containing α3 H-2K^k always had a higher mean fluorescence in comparison to other transfectants (pattern C). Therefore, we located the allodeterminants seen by these antibodies to the α3 domain of the H-2K^k antigen.

These results show that the vast majority of antibodies are strongly influenced in their binding capacity if the α1 and α2 domains of the H-2K antigen are of different alleles.

DISCUSSION

We have previously shown that CTL generated during influenza A infection recognize determinants made up of the

Table 1. Localization of serological determinants on H-2K^k and H-2K^d antigens by binding of monoclonal antibodies to cell lines expressing hybrid antigens

Alleles of K^d/ K^k hybrid antigen domains				Cell line	Binding pattern						
α1	α2	α3	MC*		A	B	C	D	E	F	G
					FACS mean values†						
				IC9	35	30	62	65	81	51	60
d	*d*	*d*	*d*	9K^d-2	82	70	156	392	405	351	78
d	*d*	*d* /	*k*	9C32-15	69	70	176	366	390	374	53
d	*d* /	*k*	*k*	9C33-12	115	115	640	605	510	90	110
d /	*k*	*k*	*k*	9C31-3	89	92	520	270	105	53	95
k	*k*	*k*	*k*	9K^k-34	465	444	505	40	41	41	30
k	*k*	*k* /	*d*	9C23-5	585	596	734	102	103	86	92
k	*k* /	*d*	*d*	9C24-6	579	569	211	68	80	450	76
k /	*d*	*d*	*d*	9C25-4	281	111	202	74	88	359	105
					Domains essential for binding						
					α1K^k	(α1 + α2)K^k	α3K^k	α1K^d	(α1 + α2)K^d	α3K^d	—
					mAbs‡ showing the binding pattern						
					H100-5.28 (12)	H116-22.7 (12)	S13.29 (13)	34-1-2S (14)	31-3-4 (14)	K9-18§	K7-65¶ (15)
					H100-27 (12)	H142-45 (12)	S13.11 (13)	20-8-4S (14)	15-5-5S (14)		
					T32-214§	H100-30 (12)		28-8-6S (14)	F35-119 (16)		
					T22-91.25§	H141-11 (12)			F35-140 (16)		
					T32-637.2§	Y-3 (17)			MN 37 (13)		
						11.4.1 (18)					
						H142.23 (12)					
						E3.25 (19)					
						3.83 (14)					
						36-7-5S (14)					
						15-3-1S (14)					
						R1-9.6 (20)					
						16-3.22 (14)					

*Membrane spanning and cytoplasmic portion.
†Values are taken from analysis with the monoclonal antibodies (mAbs) listed first in each line belonging to a certain pattern.
‡References for the mAbs are in parentheses.
§Unpublished data.
¶Monoclonal anti-H-2K^b antibody.

α1 and α2 domains of the H-2K^d and H-2K^k antigens (7). Expression of the α1 and α2 domains of the same allele was the requirement for efficient lysis of the infected cells. In the present paper we have extended our study to the allogeneic system. In addition, we have analyzed a new series of constructs (i.e., pC23, pC24, and pC25) of the *H-2K* genes. Furthermore, two of our new hybrid genes (plasmids pC26 and pC35, Fig. 1) differ only from their parental *H-2K* genes in the α2 domain. This gave us the opportunity to examine whether or not the α2 domain of the H-2K^d and H-2K^k antigens can be recognized independently of the other domains by CTL. Our results indicate that the α2 domain cannot itself constitute determinants that lead to the recognition and detectable lysis by CTL bulk cultures (Fig. 3 *E* and *F*). This is in agreement with our earlier findings as well as with data reported in the H-2K^b/D^b system (6) that the α1 domain is not recognized independently by CTL as far as this could be analyzed in bulk cultures. Therefore, the discrepancy with the earlier mentioned H-2L^d/D^d system is not resolved (5).

Using a limiting dilution system, we have shown that >90% of the individually tested T-cell clones could not lyse target cells expressing H-2K hybrid antigens with α1 and α2 domains of different alleles. Our conclusion is that the α1 and α2 domains interact with each other and generate determinants that are recognized by the majority of CTL clones; however, a few clones were found for which the α1 domain was sufficient, regardless of the allele of the neighboring domain (Fig. 4). We found only 1 of 76 CTL anti-H-2K^k clones and 11 of 71 anti-2K^d clones that recognized hybrid antigens with α1 and α2 domains of different alleles. The different frequencies could imply that the determinants on the H-2K^k domains are more influenced by the H-2K^d domain(s) than vice versa.

The analysis with monoclonal antibodies revealed that most allodeterminants are on conformational structures composed of the α1 and α2 domains. This is the case for the private specificities H-2.23 of the H-2K^k antigen (antibody 16-3-22S) and H-2.31 of the H-2K^d antigen (antibody 31-3-4S). The same is true for the public determinants seen by the two monoclonal rat antibodies R1-9.6 and MN37. The localization of allodeterminants to either the α1 domain or the α1 and α2 domains together agrees with few exceptions to our previous assignment to epitope clusters based on antibody crossblocking studies (12). None of the 29 anti-H-2K^k and anti-H-2K^d antibodies bound to the α2 domains as determined by FACS analyses with C26 and C35 hybrid-antigen-expressing cells (data not shown). Three of 29 antibodies reacted with the α3 domain on hybrid antigens with unreduced strength (Table 1). On the other hand, none of the 150 CTL clones tested recognized the α3 domain.

In conclusion our results strongly suggest that the conformational structure of the α1 and α2 domains are not independent of each other but that they are hardly influenced by α3 and the COOH-terminal part of the H-2K antigen. Exchange of the α1 and α2 domains of the H-2K antigens leads to loss of most serological and CTL determinants. It could then be expected that the interaction of the α1 and α2 domains of different alleles might generate new antigenic determinants. Direct stimulation with cells expressing such hybrid antigens might reveal such neodeterminants.

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