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Genetic Analysis of an *H-2* Mutant, B6.C-*H-2*^{ba}, Using Cell-Mediated Lympholysis: T- and B-Cell Dictionaries for Histocompatibility Determinants are Different

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Abstract

B6.C-*H-2*^{ba} [H (z1)] is a mutant derived from C57BL/6. The two strains mutually reject their skingrafts and are incompatible in the mixed leucocyte reaction (MLR) and in cell-mediated lympholysis (CML) assays. They are serologically indistinguishable.

This report shows that H(z1) carries a new, private *K* end CML specificity clearly distinguishable from that of B6 by a third party strain, HTG. Antisera directed against the private H-2K specificity of B6 present on H(z1) cells) can block CML between the two strains in either direction. The new CML specificities of H(z1) cross-react with (public) CML specificities controlled by both *K* and *D* regions of other unrelated haplotypes. The results suggest that H(z1) carries a mutation in the *H-2K* locus itself or in a closely linked gene, the product of which is also physically associated with the H-2K molecule corresponding to the cis-configuration of the alleles in both loci.

These findings indicate that T- and B-cell dictionaries for histocompatibility determinants are different.

Introduction

When a leucocyte population from peripheral blood, spleen, or lymph nodes (responder = R) is, in an in vitro mixed culture system, sensitized with leucocytes from

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a donor (stimulator = S) carrying a different haplotype at its major histocompatibility complex (MHC) then, in the responder population thymus-derived lymphocytes give rise to killer cells. These are able to recognize specifically and to lyse target cells sharing an MHC haplotype with the stimulator cell. Such systems can be viewed as candidates for in vitro models of allograft rejection (for review see Cerottini and Brunner 1973).

In studies on the genetic control of induction and effector phase of this type of cell-mediated cell lysis, the most widely used assay for cell destruction employs ^{51}Cr-labeled PHA-stimulated leucocyte populations as targets. These are exposed for not more than 4-6 hours to the putative killer cells. This particular variant of cell-mediated cell lysis is called cell-mediated lympholysis (CML) (Lightbody *et al.* 1971).

A number of reports from different laboratories show that in the mouse the determinants detected by the killer cells in CML (CML-determinants) are controlled by loci very closely linked or identical with the *H-2K* and *H-2D* loci controlling the serologically defined ubiquitous major histocompatibility antigens of this species (Alter *et al.* 1973, Abbasi *et al.* 1973, Nabholz *et al.* 1974). This interpretation is based on comparison of the results obtained with different combinations of responder, stimulator, and targets (T) involving congenic and noncongenic inbred strains carrying a variety of recombinant *H-2* haplotypes. The results suggest, but do not prove, that CML-determinants are identical with the (H-2K and H-2D) molecules that carry the classical H-2 specificities. Further evidence suggesting identity came from studies employing hyperimmune anti-H-2 sera with defined specificity (Nabholz *et al.* 1974). Such sera were found to block CML only when they are directed against the H-2K or H-2D incompatibility against which the killer is active. (In particular, sera directed against immune response region associated (Ia-) antigens have no blocking activity in CML; M. Nabholz, unpublished results).

(The only findings that seem incompatible with identity of CML, with *H-2K*, and *H-2D* determinants are those of Edidin and Henny (1973) who found that capping with polyspecific sera probably directed mainly against *H-2* antigens could remove all antigens as detected by immunofluorescence and complement mediated lysis without impairing the susceptibility of capped targets to cell-mediated lysis. Barring proof to the contrary, we feel that their data are likely to reflect different requirements of various assay systems with regard to the number of H-2 molecules on the target cell.)

If the CML loci are not identical with the *H-2K* and *H-2D* genes, then an explanation of the accumulated data requires either a) that not only the CML genes but also their products are very closely associated with *H-2K* and *H-2D* genes or products, respectively, the latter association corresponding to the coupling phase of the alleles involved; or b) that anti-*H-2* sera generally contain undetected noncomplement binding blocking antibodies directed against CML determinants.

The preceding summary of the present situation illustrates the limits of the information that can be obtained from studies with genetic recombinants in a fine structure analysis of *H-2*. Clearly the development of strains carrying novel *H-2* haplotypes derived by point mutations from a well-characterized existing haplotype could further such analysis. However, in view of our considerable ignorance of the nature and mechanisms of mutagenesis in higher organisms, it seems advisable to investigate to what extent our concepts, derived from studies in bacteria and fungi, can be extrapolated to mammals.

Bailey *et al.* (1970), through skin grafting, discovered in a (C57BL/6 × BALB/c)F_1 mouse a spontaneous mutation in the *H-2*^{b} haplotype. The novel haplotype is designated *H-2*^{ba} and carried on a C57BL/6 (B6) background in the strain B6-C-H(z1) (short: H(z1), alternative designation B6.C-*H-2*^{ba}). The mutant and B6 show strong reciprocal skin-graft rejection, and the mutation responsible for this incompatibility could, by complementation studies, be mapped into the *K* end of the *H-2* complex. Nevertheless, the strains could not be distinguished serologically either by H-2 typing or by repeated attempts at reciprocal immunization (Bailey *et al.* 1970). Nor did a ratanti-B6 serum reveal any serological differences with H(z1) (M. Cherry and D. Bailey, personal communication). Widmer *et al.* (1973) demonstrated strong bidirectional incompatibility with regard to both the mixed leucocyte reaction (MLR) and CML. This latter result, confirmed by us, presents a puzzle in that previous studies in various laboratories (Bach *et al.* 1972, Meo *et al.* 1973a, Nabholz *et al.* 1974, Alter *et al.* 1973, Abbasi *et al.* 1973) had provided strong evidence for genetic separation of CML- and MLR-determinants, a strong MLR-response being tied to incompatibiltiy within a segment of the *I*-region (Meo *et al.* 1973b) not including *H-2K*.

Therefore, both with regard to an understanding of mutational events in mammals, in general, as well as in the context of the fine structure analysis of the MHC, an attempt to map more precisely the mutational changes in H(z1) leading to MLR- and CML-incompatibility with B6, respectively, seemed necessary. The present report contains results bearing on the location of the CML mutation in H(z1) as well as those of a study on cross-reactions between the new (private) CML specificity of H(z1) and (public) CML specificities of other *H-2* haplotypes.

Materials and Methods

Mice. Animals of the strains C57BL/6J (B6) and BALB/cJ as well as the F_1 hybrids, B6 × A/J and BALB/c × B6, were obtained from the Roche animal colony in Füllinsdorf. All other inbred strains are maintained as breeding stocks in the Human Genetics Department, Ann Arbor. For production, they are occasionally bred in the Basel Institute for Immunology. The original source of most of these strains is listed in a previous publication (Meo *et al.* 1973a). Strain B6.C-H(z1) was a gift of Donald W. Bailey, The Jackson Laboratory; strain AQR was a gift of Jan Klein, present address: Department of Microbiology, The University of Texas, Southwestern Medical School, Dallas, Texas.

H-2 haplotypes of strains are indicated in terms of the allelic origins of the *K, I, S,* and *D* regions respectively.

All experimental methods have been described in detail in a previous report (Nabholz *et al.* 1974). Briefly, they are as follows.

Culture Conditions. Cells are cultured at 37°C in a humidified incubator supplied with a mixture of 95% air-5% CO_2.

Media. Culture medium is RPMI containing penicillin, streptomycin, additional glutamine, 30 mM HEPES, 3×10^{-5} M 2-mercaptoethanol, and 20% fetal calf serum.

CML assays are carried out in similar medium, but without 2-mercaptoethanol and with only 10% fetal calf serum.

Mixed Leucocyte Cultures (MLC). To generate killer cells 10^7 responder cells (spleen) are cocultured for 5 days with 8×10^6 irradiated (3300 r) stimulator cells (spleen) in 4.5 ml medium in a 30-ml plastic culture flask (Falcon No. 3012) standing on its side.

Targets. Target cell cultures (spleen) are set up on the same day as the MLC's. PHA (final conc. 1:100 stock solution of Difco PHA-M) is added on day 2. Cells are harvested on day 5, labeled for 3 hours with ^{51}Cr (500μCi in. 3 ml containing 5-10 $\times$ 10^6 cells), and washed twice. Viable cells are separated on a Ficoll-Urovison gradient and washed once more.

CML-assay. The MLC's are spun down and the cells resuspended to a constant volume so that each 150μl contain the descendants of 10^6 or 2×10^6 responder cells. Two threefold serial dilutions are made. Targets are adjusted to a concentration of, usually, 2×10^4 cells/50μl. One hundred-fifty microliters of killer cell suspension and 50μl target cell suspension are added to the well of a round-bottomed microtest plate. Killing activity of each suspension is assayed in triplicate. The mixtures are spun gently for 2.5 minutes. After incubation for 4 hours at 37°C the plates are centrifuged again, 100μl of the supernatant are collected from each well, and 100μl of a 10% Zaponin (Coulter Electronics) solution added to each well. After further incubation another 100 μl are harvested.

In experiments involving the addition of antisera to killer/target mixtures, 10μl of antiserum are added to the well first, then 50μl of killer cell suspension, and last, 25μl of target cells. The microtest plate is kept on ice during the addition of cells and sera and not spun before incubation at 37°C. One hundred microliters of PBS are added before the postincubation spin. The ^{51}Cr counts in the two samples obtained from each well are determined and used to calculate percent specific release = 100 $\times$ (percent experimental release – percent spontaneous release) / (100 – percent spontaneous release) and standard deviations thereof.

Antiserum Fractionation: In one experiment ammonium sulfate precipitates of antisera were used. The precipitates had been redissolved in, and extensively dialyzed against, phosphate buffered saline.

Results

Mapping the New Private CML Specificity Characteristic of B6.C-H-2^{ba}. a) Cross-reaction Between B6 and H(z1). When B10.A (5R) responder cells (*H-2* haplotype: *bbdd*) are stimulated with B6 cells, the resulting killer cells display approximately equal levels of lytic activity against B6, H(z1) or A.BY targets (the last strain shares with B10 the *H-2^b* haplotype) (Fig. 1). This is expected as the killer cells should be directed against the private CML specificity associated with the H-2D^b region common to all three target strains. The specific lysis observed in this situation can be used as a standard to which lytic potentials of other responder/stimulator combinations on the same target cells are compared (see also Nabholz *et al.* 1974). Thus when cells from strain HTG (dddb) are stimulated with B6, the resulting killer cells are much more active against B6 and A.BY than against H(z1). The reverse result is obtained when H(z1) is the stimulator of HTG. These results establish that the mutation(s) in H(z1) must have led to a change in the private K end CML specificity of B6, which,

by extrapolation from the earlier described work, is controlled by the H-2K^b allele or by a gene extremely closely linked to it.

b) Inhibition of CML Between B6 and H(z1) by Specific Antisera. Previous work (Nabholz *et al.* 1974) had demonstrated that antisera could block CML only when they were directed, presumably, against the same H-2K or H-2D determinants as the killer cells. [Anti-H-2 sera directed against the responder cells have no detectable blocking activity, (Cerottini and Brunner 1973, Nabholz *et al.* 1974)]. In particular, killing directed against K^b-region CML determinants could be blocked by an anti-H-2.33 serum [(B10.D2 × A)F_1 anti-HTI]. This inhibition did not interfere with killing directed against *D* end determinants on the same target cells. This is true even when both killer and target cell carry the K^b region (unpublished results). The same serum was able to completely block lysis of both B6 and H(zl) targets by HTG (*dddb*)

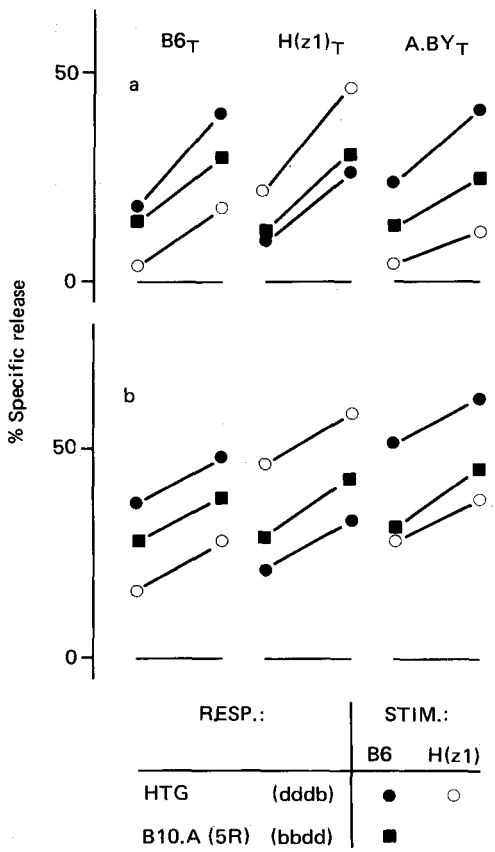

Fig. 1. Cross-reactions in CML between B6, H(z1) and A.BY: The allelic origin of the *K, I, S* and *D* regions of the *H-2* complex are indicated for HTG and B10.A (5R). Subscript T refers to targets. **Ordinate:** To each well the killer cells derived from a known number of responders are added, Number of responders/well: **Exp. a:** 0.11 × 10^6 and 0.33 × 10^6; **Exp. b:** 0.17 × 10^6 and 0.50 × 10^6. Total ^{51}Cr cpm and percent spontaneous release of targets (2 × 10^4): **Exp. a:** B6: 5252 cpm, 35%, H(z1): 4805 cpm, 26%, A.BY: 4667 cpm, 20%; **Exp. b:** B6: 31339 cpm, 48% H(z1): 24958 cpm, 44%, A.BY: 23390 cpm, 38%.

cells stimulated with either strain (Table 1). In this study, an additional serum (a gift from S. Cullen and S. Nathenson) prepared by immunization of HTG mice with cells from a B6 thymoma cell line, EL4, which does not express detectable Ia- (immune response region associated) antigens, was used. The difference between the two sera lies in the fact that the anti-H-2.33 serum may, and probably does, contain anti-Ia antibodies (for review, see Shreffler and David 1974), while the latter, in precipitation analysis (Cullen *et al.* 1974), does not recognize Ia-specificities (S. Cullen and S. Nathenson, personal communication). It may, on the other hand, have activity against some of the public H-2K^b specificities.

High concentrations of these two sera completely abrogate specific cell lysis either by appropriately sensitized H(z1) cells against B6 targets or in the reverse direction (Fig. 2a). Sodium sulfate fractionated antisera still give very strong inhibition (Fig. 2b). The degree of inhibition can be assessed by comparing the specific lysis obtained in the presence of antiserum to the dose-response curve resulting from variation of the killer cell number. Thus, e.g., in the experiment shown in Fig. 2b, the highest concentration of either fractionated antiserum reduces the specific release of B6 stimulated with and tested against H(z1) to the level obtained with a 1:9 dilution of the same killer, i.e., killing is inhibited by about 90%.

In both experiments inhibition by either antiserum of B6 target cell lysis is significantly stronger than inhibition in the reverse direction. The results suggest the possibility that the affinity of blocking antibodies in these sera is somewhat less for H(z1) than for B6. However, as we do not know the kinetics of inhibition with regard to antisera concentration at different concentrations of killer cells, we cannot interpret this observation with certainty. The cytotoxicity titers of the two antisera on B6 and H(z1) cells are not significantly different.

Table 1. Inhibition of CML Against *K* end CML Determinants of B6 and H(z1) with an Anti-H-2.33 Serum[a]

Responder: HTG (*dddb*)				
Stimulator	No. of responders ($\times 10^{-6}$)	Serum (dilution)[c]	Target	
			B6	H(z1)
B6	1.0	–	36 ± 3	20 ± 0
	.33	–	28 ± 3	6 ± 4
	.11	–	19 ± 2	−1 ± 9
	1.0	1:17	−5 ± 4	−5 ± 3
	1.0	1:68	1 ± 2	−4 ± 1
H(z1)	1.0	–	21 ± 3	38 ± 4
	.33	–	14 ± 1	28 ± 5
	.11	–	7 ± 2	22 ± 2
	1.0	1:17	1 ± 0	−3 ± 5
	1.0	1:68	7 ± 0	9 ± 4

[a](B10.D2 × A)F_1 anti-HTI.
[b]*H-2* haplotype of HTG in terms of allelic origins of *K, I, S* and *D* regions.
[c]Final dilution of serum in assay mixture.
Data are expressed as percent specific release (see Materials and Methods)

Analysis of Cross-reaction Between the New CML-specificity of H(z1) and CML-Specificities of Other H-2 Haplotypes. Widmer *et al.* (1973) had reported that B6 stimulated with H(zl) was able to lyse also B10.BR (H-2^k) cells to a considerable degree. Our own work hinted at the existence of cross-reactions detectable in CML (Nabholz *et al.* 1974) and subsequent analysis (manuscript in preparation) has revealed extensive but specific cross-reactivity in CML assays between different *H-2* haplotypes, including cross-reaction between *K* and *D* end CML specificities.

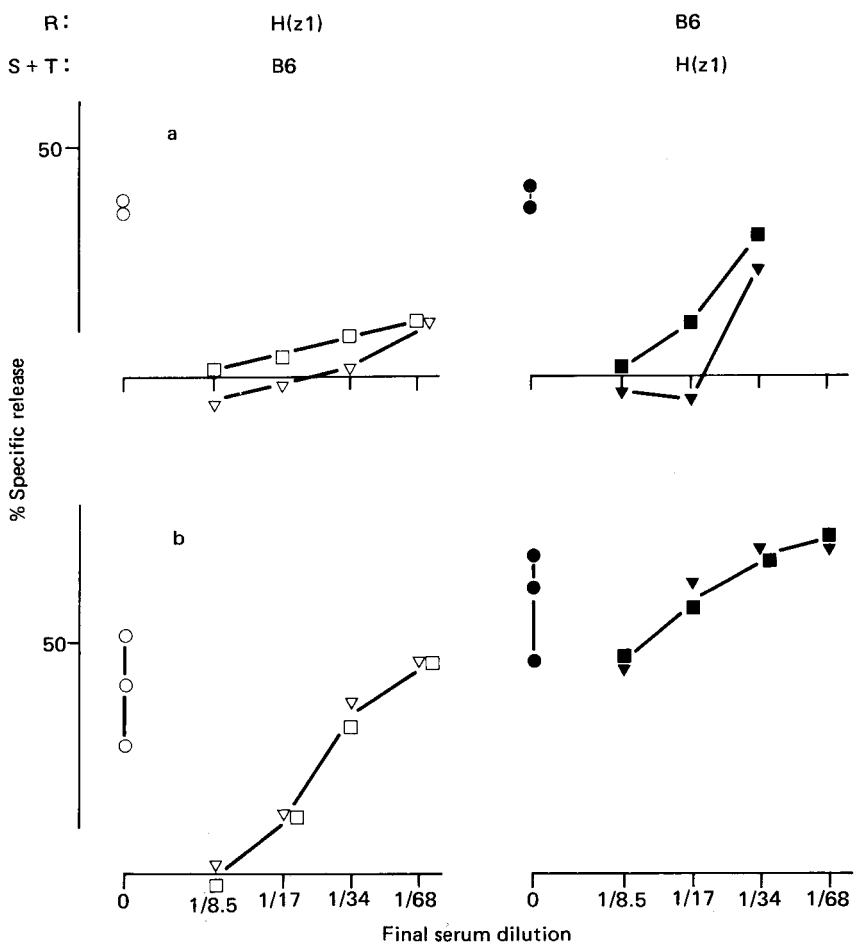

Fig. 2. Inhibition of CML between B6 and H(z1) by anti-*H-2* sera: **Exp. a:** complete sera; b: sodium sulfate fractionated sera. In both experiments a control for antibody dependent cell-mediated cell lysis was negative. O, ●: Reaction without serum at killer cell concentrations corresponding to 10^6, 0.33 × 10^6 and 0.11 × 10^6 (only exp. b) responders/well. All reactions in the presence of antisera involve the highest killer cell concentration (10^6 responders). □, ■: Reaction in presence of anti-H-2.33 serum. △, ▲: Reaction in presence of anti-EL4 serum. Targets: (cpm, percent spontaneous release). **Exp. a:** (1.25 × 10^4 cells): B6: 14604 cpm, 55%; H(z1): 12728 cpm, 54%. **Exp. b:** (1.5 × 10^4 cells): B6: 6694 cpm, 45%; H(zl): 6645 cpm, 38%.

We have attempted to map some of the cross-reacting determinants detected by B6 when stimulated with B6.C-H(z1). The results of one particular experiment are illustrated in Fig. 3 and our findings are summarized in Table 2.

It is clear that B6 stimulated with H(z1) reacts significantly against all tested strains except HTG. Comparing the *H-2* haplotypes of HTG (*dddb*), HTI (*bbbd*), and BALB/c (*dddd*) it is evident that at least one cross-reacting determinant is controlled by the *H-2D*^{d} region. This cross-reaction is abrogated, as expected, by complementation with either A/J or BALB/c. Furthermore, although there is significant cross-reaction by B6 stimulated with H(z1) against B10.A (2R) (*kkdb*), it is much weaker than against B10.A (*kkdd*). This suggests that part of the latter cross-reaction is against determinants controlled by the *K* end of the *H-2*^{a} haplotype. This interpretation is supported by the fact that complementation with BALB/c reduces the cross-reaction against B10.A but not against B10.A (2R). That this cross-reacting specificity is controlled by the *H-2K*^{k} region follows from the fact that it is not present on cells of the strain AQR (*qkdd*).

Discussion

The results reported here establish that H(z1) carries a new private CML specificity controlled by the gene determining the private *K* end CML specificity of B6. Thus, from the previous genetic analyses of CML, we can extrapolate that the private CML specificity characteristic for H(z1) is controlled by a locus identical or very closely linked to *H-2K*.

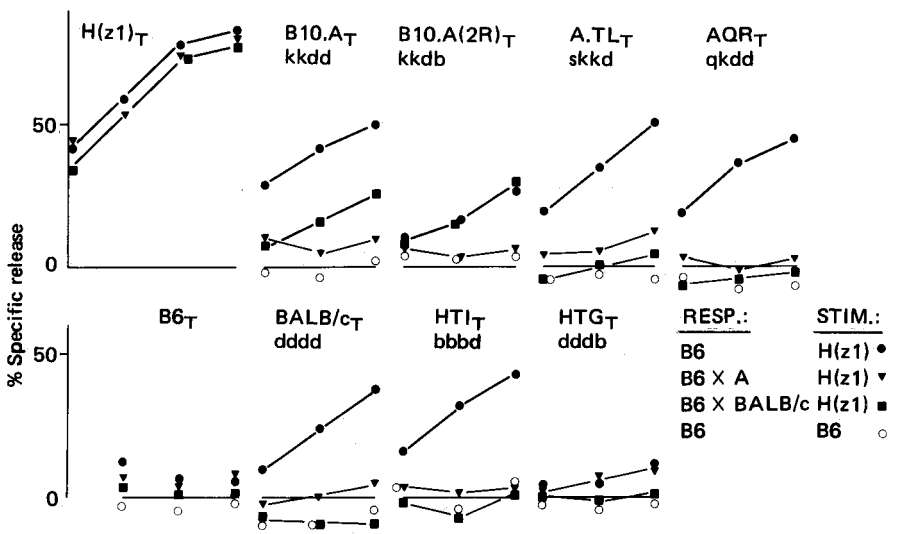

Fig. 3. Cross-reactions in CML between *H-2*^{ba} and other *H-2* haplotypes. For each target (subscript T) the allelic origin of the *K, I, S,* and *D* regions of the *H-2* complex are indicated. Killers are tested at concentrations corresponding to 2.2 × 10^6, 0.7 × 10^6, and 0.2 × 10^6 responders/well. Targets (2 × 10^4/well): cpm, percent spontaneous release: B6: 4926 cpm, 29%; H(z1): 5970 cpm, 33%; HTG: 8232 cpm, 26%; BALB/c: 8142 cpm, 28%; B10.A (2R): 10246 cpm, 25%; B10.A: 6160 cpm, 40%; HTI: 7616 cpm, 40%; A.TL: 6691 cpm, 26%; AQR: 5324 cpm, 43%.

Table 2. Cross-reaction in CML Between *H-2*^{ba} and Other *H-2* Haplotypes

RESP.[a]	STIM.	Target[a] B6 (*bbbb*)	H(z1)	HTI (*bbbd*)	HTG (*dddb*)	BALB (*dddd*)	B10.A (2R) (*kkdb*)	B10.A (*kkdd*)	AQR (*gkdd*)
B6 (*bbbb*)	H(z1)	–	++++	++	±	++	+	++	++
(B6 × A)F$_1$ ($\frac{bbbb}{kkdd}$)	H(z1)	–	++++	–	±	±	–	–	–
(BALB/c × B6)F$_1$ ($\frac{dddd}{bbbb}$)	H(z1)	–	++++	–	–	–	+	+	–

[a]*H-2* haplotypes are given in terms of allelic origins of *K*, *I*, *S* and *D* regions.

±: Negative or very weak reaction.

+, ++, ++++: increasing amounts of lysis (see Fig. 3).

This interpretation is supported by the finding that both a monospecific H-2.33 serum as well as the HTG-anti-EL4 serum, can block the lytic reaction between B6 and H(z1) in either direction. Together the experiments suggest that the new private CML specificity of H(z1) is controlled by the *H-2K* locus itself.

Two alternative explanations cannot be ruled out: a) The CML determinants may in fact be controlled by a locus not only very closely linked to the *H-2K* locus but the product of which also is on the cell surface physically linked to the *H-2K* gene product, recognized by the antisera. b) It is conceivable that the private *K* end CML determinants of B6 and H(z1) are controlled by genes of the *I* region (as the earlier genetic analyses of CML made this very unlikely for the available haplotypes resulting from crossovers between *K* and *I* regions, this would probably mean that CML determinants can be controlled by different loci in the different haplotypes and that the anti-EL4 serum contains noncomplement fixing antibodies against these determinants).

The cross-reaction analysis clearly demonstrates the existence of CML specificities shared by *K* and *D* end associated CML determinants, and that the cross-reacting specificities analysed so far map in the *K* and *D* regions themselves. The finding of *K-D* cross-reaction between a presumably novel CML specificity arisen by mutation and specificities controlled by existing *K* and *D* alleles suggests that such cross-reactions do not necessarily reflect the very "old" specificities that arose prior to the duplication event(s) that gave rise to the bipartite structure of *H-2* (Klein and Shreffler 1970).

If the mutation is in the *H-2K* locus then, not being detectable serologically, it presumably affects only a small part of the *H-2* molecule. [One would then interpret the cross-reactions as an example of a "simplex-complex" situation, i.e., the cross-reactions are a result of similarities between the cross-reacting specificities and the new private CML specificity of H(z1)].

In any case the alteration must be in the site determining the private CML specificity of B6 and, presumably, the specificity responsible for the strong skin-graft rejection between B6 and H(z1). Thus, if the size of the T-cell receptor combining site is comparable to that of an antibody molecule, then the results suggest that the private CML-(T cell) and the private serological (B cell) specificities are not the same molecular sites, whether they are on the same molecule or not. Alternatively, one could envisage that the structure recognized by the T cell is much larger than that recognized by an antibody and that the unchanged private B cell specificity is a part of the private T cell specificity, but the only serologically recognizable one. Whatever the view on the nature of the T-cell receptor for histocompatibility determinants, the data reported here suggest that, as for soluble antigens (Hammerling and McDevitt 1974), T- and B-cell dictionaries for histocompatibility determinants are different.

The results reported here do not resolve the puzzle presented by the observation that B6 and H(z1) are mutually incompatible with regard to both MLR and CML, while the determinants recognized by the two tests have genetically been separated and mapped into different parts of the *K* end of the *H-2* complex using two different recombinants between *H-2K* and *Ir-1A* (Alter *et al.* 1973, Meo *et al.* 1973a, Nabholz *et al.* 1974, Abbasi *et al.* 1973). The most plausible explanations of this apparent inconsistency are:

1. The $H\text{-}2^b$ haplotype is different from the previously analysed ones (*k,s,q*) with

regard to the distribution of MLC genes in that the *H-2K*^{b} locus (or the CML locus associated with it) controls a strong MLR determinant. This hypothesis is supported somewhat by the finding that another *H-2*^{b} mutant derived from B6, M505, although different from H(z1), is incompatible with B6 both in MLR and CML (see accompanying paper by Forman and Klein). Alternatively, one might argue that the private *K* end CML specificity of B6 is controlled by the strong MLR determinant(s) in the *I* region, but the evidence suggesting that in the test described here, i.e., on PHA-stimulated target cells, no determinants controlled by *I* region loci are detected is quite strong. (On the other hand, recent experiments have, in fact, revealed that *I* region-associated incompatibilities can be detected through skin grafting (Klein *et al.* 1974) and by killer cells generated in vitro when LPS-stimulated, instead of PHA-stimulated cells, are used as targets (M. Nabholz, *et al.* manuscript in preparation)).

2. According to the grid hypothesis (Boyse 1970, Ceppelim, 1970), a change in the *H-2K* locus could affect the expression of MLR determinants coded for by genes in the *I* region. There is no evidence supporting an interaction of this type, and earlier cited genetic analyses of MLR show that it is unlikely to be operative at least in the available relevant recombinants between *H-2K* and *Ir-1*.

3. H(z1) differs from B6 not only in the *H-2K* but also in the *I* region-associated MLR-genes because of a second mutation that has occurred in the *H-2*^{ba} chromosome. [No differences between B6 and H(z1) have been found in responsiveness to a number of antigens, the response to which is under *Ir-1* control (McDevitt, personal communication)]. The similarities between H(z1) and M505 mentioned above would lead us to expect that the probability of such a second mutation leading to MLR incompatibility is not independent of the one detected by CML.

Further genetic analysis of the MLR incompatibility between B6 and H(z1) may help to distinguish between these alternatives, although most likely the final answer will depend on a biochemical characterization of the MHC products involved.

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