Brief Definitive Report

CELL-MEDIATED LYMPHOLYSIS

IMPORTANCE OF SEROLOGICALLY DEFINED *H-2* REGIONS*

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(Received for publication 7 February 1973)

The major histocompatibility complex (MHC) in the mouse strongly influences allograft rejection. This complex can be divided into four regions separable by genetic recombination (1, 2). Two regions, *H-2K* and *H-2D*, at the left and right ends of the complex, respectively, contain loci controlling serologically detectable (SD) antigens. An immune response (*Ir*) region, next to *H-2K*, includes genetic loci determining immune responsiveness (3, 4); between *Ir* and *H-2D* is a fourth region marked by the *Ss-Slp* locus (5).

Two in vitro tests can be used to detect differences at the MHC between different inbred mouse strains. Mixed lymphocyte cultures (MLC) measure the proliferative response of lymphocytes of one strain as they react to histocompatibility differences of a second strain (6). Responding cells activated in MLC to allogeneic MHC antigens can lyse ^{51}Cr-labeled target lymphocytes in the cell-mediated lympholysis (CML) assay (7). Recent studies suggest that MHC differences that have not been defined serologically are primarily responsible for lymphocyte activation in MLC (8). We have called such differences "lymphocyte defined" (LD) to contrast them with the SD differences associated with the *H-2K* and *H-2D* regions. The LD differences primarily map between *H-2K* and *H-2D*. Lymphocyte-defined differences of the MHC probably also exist in man (9–12). Recently Eijsvoogel et al. demonstrated in one family in man that these presumed LD differences, which lead to MLC activation, were not sufficient to serve as a "target" in CML; thus for significant CML to occur it was necessary to include the SD differences on the target cells. Further, only one of

* Supported by National Institutes of Health grants GM-18314, DE-02731, AI-08439, GM-15422, and AI-06525; National Foundation-March of Dimes grant CRBS 246; and Office of Naval Research grant N000-67-A-128-0003. This is paper no. 1,619 from the Laboratory of Genetics, University of Wisconsin, Madison, Wis. 53706.

‡ D.J.S. is an NIH Trainee supported by National Institute of General Medical Sciences grant GM-00398.

§ M.L.B. is a recipient of the American Cancer Society Faculty Research Award.

the SD loci of *HL-A* (the Four locus region) appeared to serve as a target for CML (13). The need for SD differences for CML was likewise suggested by the studies of Trinchieri et al. (14).

Our present studies in mouse further investigate the role of LD and SD differences in CML. In certain strain combinations that are SD identical but different for LD (i.e. there is MLC reactivity) there is no CML. However, the presence of either an *H-2K* or an *H-2D* region difference (thus including an SD difference) is sufficient for significant CML to occur.

Materials and Methods

Mice used in these studies are raised in our own colonies. These strains differ for various regions of the MHC and have been discussed in detail (8). All strains used, except AQR,[1] are congenic, i.e., they are genetically identical except for differences in the MHC. However the effects seen with AQR in MLC seem attributable to the MHC (8). We refer to the four regions on the MHC chromosome (*H-2K*, *Ir*, *Ss*, and *H-2D* from left to right) with four capital letters, one for each of the four regions. For instance, strain B10.A, which carries the $H\text{-}2^a$ chromosome, is designated *KKDD*; it has the *H-2K* and *Ir* regions derived from an $H\text{-}2^k$ chromosome, and the *Ss* and *H-2D* regions derived from an $H\text{-}2^d$ chromosome. (Lower case superscripts refer to the different *H-2* chromosomes; capital letters, which are not written as superscripts, to the various regions.) Strain B10.A(2R) is derived from an $H\text{-}2^a/H\text{-}2^b$ heterozygous animal after a recombinational event between *Ss* and *H-2D*. The B10.A(2R) (*KKDB*) animal has the *H-2K*, *Ir*, and *Ss* regions derived from the $H\text{-}2^a$ chromosome, and the *H-2D* region derived from the $H\text{-}2^b$ chromosome. B10.A and B10.A(2R) thus differ only for *H-2D*; B10 and B10.A(2R) differ for *H-2K*, *Ir*, and *Ss* but are identical for *H-2D*. In some cases, since the chromosomes in question are derived from the same heterozygous genotype, we can be certain that two regions carrying the same designations are identical; in other cases we cannot be sure of identity by genetic derivation and rely on phenotypic identity, for serological factors for instance.

MLC tests are done using the micromethod of Widmer et al. (15). All cells are cultured in RPMI 1640 (Grand Island Biological Company, Grand Island, N. Y.), supplemented with penicillin, streptomycin, and 5% heat-inactivated human plasma. Stimulating cells are treated with mitomycin C. Cultures used for assay of MLC reactivity are labeled with tritiated thymidine for 16–18 h 3 days after initiation of culture.

Effector cells, to be used in CML, are obtained from MLC after 88–90 h of incubation. These cells are suspended at 1×10^7 viable cells/ml in RPMI 1640 with 5% heat-inactivated fetal calf serum.

Target cells for CML are prepared from lymph nodes and incubated in RPMI 1640 with 5% fetal calf serum. 3 days before use they are stimulated with phytohemagglutinin. The cells are labeled with 250 μCi of $Na_2[^{51}Cr]O_4$ (New England Nuclear, Boston, Mass.) at 37°C in 5% CO_2 for 60 min. After three washes they are adjusted to a concentration of 1×10^5 viable cells/ml in RPMI 1640 with 5% heat-inactivated fetal calf serum.

For the CML assay, 0.75×10^6 to 1×10^6 effector cells are incubated with 1×10^4 target cells in Linbro round-bottom microtiter plates (no. IS-MRC-96TC). After 3 h of incubation at 37°C, 0.05 ml of 5% fetal calf serum in RPMI 1640 is added to each well and the cells are centrifuged in the plates. A constant aliquot of the supernatant fluid is aspirated from each well and the amount of radioactivity released in the supernatant fluid determined. The maximum release (MR) value of each target cell preparation is measured by determining the

[1] Klein, J. Unpublished observations.

amount of ^{51}Cr released into the supernatant fluid by 1×10^4 cells after repeated freeze-thawing. Spontaneous release (SR) of ^{51}Cr from the same number of target cells, incubated in medium alone for 3 h, is also measured. Quantitation of the test is expressed as percent (% ^{51}Cr release) of (net experimental release [ER] − spontaneous release [SR]) divided by (net total release [MR] − [SR]), as given by the formula (ER − SR/MR − SR) × 100. All assays are done in triplicate.

RESULTS AND DISCUSSION

The two strain combinations [AQR–B10.T(6R) and B10.A(4R)–B10.A(2R)] of greatest interest in these studies have LD differences associated with MLC

TABLE I

MLC and CML with SD-Identical, LD-Different Combinations

	MLC sensitization			CML assay		
	MLC	Responding cell (effector)	Stimulating cell (sensitizing)	Target cell	^{51}Cr released	CML
	mean cpm ± SD				*mean cpm ± SD*	%
A*	45,841 ± 1,921	AQR (*QKDD*)‡	B10.T(6R) (*QQQD*)	AQR (*QKDD*)	489 ± 51	−5.1
				B10.T(6R) (*QQQD*)	558 ± 50	2.2
	63,491 ± 4,511	B10.T(6R) (*QQQD*)	AQR (*QKDD*)	B10.T(6R) (*QQQD*)	471 ± 7	5.0
				AQR (*QKDD*)	475 ± 23	−6.0
	49,763 ± 1,726	AQR (*QKDD*)	C57BL/10 (*BBBB*)	AQR (*QKDD*)	690 ± 41	9.5
				C57BL/10 (*BBBB*)	1,158 ± 66	66.9
	70,687 ± 3,425	B10.T(6R) (*QQQD*)	B10.A(2R) (*KKDB*)	B10.T(6R) (*QQQD*)	716 ± 18	15.3
				B10.A(2R) (*KKDB*)	2,004 ± 58	74.5
	64,048 ± 1,846	C57BL/10 (*BBBB*)	AQR (*QKDD*)	C57BL/10 (*BBBB*)	709 ± 20	7.3
				AQR (*QKDD*)	1,749 ± 114	86.5
	52,782 ± 1,534	C57BL/10 (*BBBB*)	B10.T(6R) (*QQQD*)	C57BL/10 (*BBBB*)	706 ± 6	7.0
				B10.T(6R) (*QQQD*)	1,565 ± 151	85.4
	12,327 ± 1,901	AQR (*QKDD*)	AQR (*QKDD*)	AQR (*QKDD*)	436 ± 31	8.9
	15,101 ± 529	B10.T(6R) (*QQQD*)	B10.T(6R) (*QQQD*)	B10.T(6R) (*QQQD*)	488 ± 35	−3.6
	14,843 ± 3,858	C57BL/10 (*BBBB*)	C57BL/10 (*BBBB*)	C57BL/10 (*BBBB*)	438 ± 14	−28.5
B§	18,729 ± 3,810	B10.A(4R) (*KKBB*)	B10.A(2R) (*KKDB*)	B10.A(4R) (*KKBB*)	468 ± 25	−4.6
				B10.A(2R) (*KKDB*)	469 ± 8	−5.5
	98,911 ± 2,753	B10.A(4R) (*KKBB*)	C57BL/10 (*BBBB*)	B10.A(4R) (*KKBB*)	747 ± 62	27.0
				C57BL/10 (*BBBB*)	1,345 ± 30	74.1
	44,163 ± 5,606	C57BL/10 (*BBBB*)	B10.A(2R) (*KKDB*)	C57BL/10 (*BBBB*)	697 ± 38	3.8
				B10.A(2R) (*KKDB*)	1,184 ± 17	62.2
	6,430 ± 2,204	B10.A(4R) (*KKBB*)	B10.A(4R) (*KKBB*)	B10.A(4R) (*KKBB*)	437 ± 52	−8.2
	18,057 ± 2,815	C57BL/10 (*BBBB*)	C57BL/10 (*BBBB*)	C57BL/10 (*BBBB*)	578 ± 45	−9.1

* The percent CML is based on the following spontaneous release (SR) and maximum release (MR) values (mean of triplicates ± SD) for each target cell: AQR, SR = 558 ± 6 MR = 1,935 ± 53; B10.T(6R), SR = 531 ± 35 MR = 1,742 ± 94; C57BL/10, SR = 653 ± 56 MR = 1,408 ± 81; B10.A(2R), SR = 621 ± 77 MR = 2,476 ± 179.

‡ The four capital letters (i.e., QKDD) refer to the various regions of the MHC as explained in the text.

§ The percent CML is based on the following spontaneous release (SR) and maximum release (MR) values (mean ± SD of triplicates) for each target cell: B10.A(4R), SR = 509 ± 42 MR = 1,388 ± 116; B10.A(2R), SR = 527 ± 29 MR = 1,582 ± 11; C57BL/10, SR = 662 ± 28 MR = 1,585 ± 83.

activation (8) and graft-vs.-host reactions,[2] but are SD identical. Table I shows the results of MLC and CML tests in these combinations. Despite significant MLC activation, AQR effector cells do not lyse B10.T(6R) target cells nor do B10.T(6R) effector cells lyse AQR target cells. Both AQR and B10.T(6R) cells are capable of mediating CML when sensitized and tested against target cells that differ in several regions of the MHC; likewise these cells are extensively lysed when used as target cells in combinations differing

TABLE II

MLC and CML with SD-Different Combinations

MLC sensitization			CML assay		
MLC	Responding cell (effector)	Stimulating cell (sensitizing)	Target cell	^{51}Cr released	CML
mean cpm ± SD				*mean cpm ± SD*	%
A* 14,819 ± 1,406	B10.A (*KKDD*)	AQR (*QKDD*)	B10.A (*KKDD*)	469 ± 11	−4.6
			AQR (*QKDD*)	372 ± 36	−3.6
44,777 ± 5,237	B10.A (*KKDD*)	B10.T(6R) (*QQQD*)	B10.A (*KKDD*)	484 ± 21	−1.4
			B10.T(6R) (*QQQD*)	534 ± 19	37.5
			AQR (*QKDD*)	623 ± 19	42.6
			C57BL/10 (*BBBB*)	383 ± 33	13.8
14,487 ± 846	B10.A (*KKDD*)	B10.A (*KKDD*)	B10.A (*KKDD*)	417 ± 54	−15.4
B‡ 13,725 ± 2,236	B10.A(2R) (*KKDB*)	B10.A (*KKDD*)	B10.A(2R) (*KKDB*)	532 ± 142	−4.9
			B10.A (*KKDD*)	552 ± 13	5.3
79,236 ± 6,902	B10.A(2R) (*KKDB*)	B10.D2 (*DDDD*)	B10.A(2R) (*KKDB*)	889 ± 67	24.9
			B10.D2 (*DDDD*)	1,541 ± 140	72.3
			B10.A (*KKDD*)	1,041 ± 32	78.5
			C57BL/10 (*BBBB*)	1,232 ± 43	40.6
10,423 ± 623	B10.A(2R) (*KKDB*)	B10.A(2R) (*KKDB*)	B10.A(2R) (*KKDB*)	434 ± 7	−13.1

The percent CML is based on the following spontaneous release (SR) and maximum release (MR) values (mean of triplicates ± SD) for each target cell.

* B10.A, SR = 491 ± 20 MR = 974 ± 61; AQR, SR = 391 ± 24 MR = 935 ± 45; B10T(6R), SR = 328 ± 25 MR = 877 ± 66; C57BL/10, SR = 325 ± 19 MR = 749 ± 22.

‡ B10.A(2R), SR = 591 ± 23 MR = 1,786 ± 60; B10.A, SR = 516 ± 14 MR = 1,184 ± 59; B10.D2, SR = 684 ± 47 MR = 1,869 ± 34; C57BL/10, SR = 721 ± 73 MR = 1,978 ± 30.

in all four regions of the MHC. Similar results are obtained when B10.A(4R) cells are tested in combination with B10.A(2R) cells. Despite MLC activation there is no CML.

Table II shows the results of experiments testing whether differences at either end of the MHC can lead to CML. In the first combination (B10.A–AQR) the effector and target cells differ for the *H-2K* region, but are identical for the *Ir*, *Ss*, and *H-2D* regions. There is no MLC activation nor CML in this combination. However, when B10.A is sensitized against B10.T(6R), which differs for the *H-2K*, *Ir*, and *Ss* regions, one finds strong MLC activation and significant CML directed at the B10.T(6R) target cell. These B10.A effector

[2] Livnat, S., J. Klein, and F. H. Bach. 1973. Graft versus host reaction in strains of mice identical for the serologically defined H-2 antigens. Manuscript submitted for publication.

cells are also cytotoxic to AQR target cells. Therefore, if the effector cells are properly activated the *H-2K* region difference alone is a sufficient target for CML.

Similarly, when one sensitizes the effector cell population against only an *H-2D* region difference [B10.A(2R)–B10.Am], there is neither MLC activation nor CML (Table II B). The B10.A(2R)–B10.D2 combination leads to strong MLC activation and CML; CML occurs also on the B10.A target cells, which differ from the B10.A(2R) effector cells at only the *H-2D* region. Less CML occurs if the effector cell is confronted with a target cell carrying a different *H-2K* or *H-2D* region.

Our present studies confirm the observation in man that in some instances an LD difference is sufficient to give MLC activation but not sufficient to allow CML. Those combinations with SD differences and MLC activations lead to CML whether the SD differences are associated with the *H-2K* region or the *H-2D* region. This contrasts with the findings in man where differences at one of the two SD loci appear to play the predominant role in CML (13).

Whether it is the serologically defined antigens themselves that are the targets for CML must be left open for further investigation. We have noted that significant CML occurs in the C57BL/6–H(z1) combination (16). These strains differ by a spontaneous mutation in the MHC, which leads to reciprocal MLC activation, skin graft rejection, and CML. Although it is possible that C57BL/6 and H(z1) differ by antigens that can be defined serologically, no such differences have been detected to date (17).

There are at least two models that can explain the apparent dichotomy between the lack of CML in the AQR–B10.T(6R) and B10.A(4R)–B10.A(2R) combinations and significant CML in C57BL/6–H(z1). The first model is that SD differences are in fact the targets for CML; C57BL/6 and H(z1) may actually differ serologically. If such an SD difference exists, it is not detected as measured by antibody production after cross-immunization between the two strains but is recognized in CML after MLC between the two strains. This could reflect the greater ease with which the same antigen triggers T cells in MLC compared with triggering of B cells for antibody production; alternatively it could indicate a qualitative difference, e.g., that the T cells recognize differences that B cells cannot recognize. The second model hypothesizes that there are two types of LD differences. One type controls the "activation" of cellular "proliferation" as measured in MLC; the second determines the target molecules detected in CML. The "LD target" differences are determined by genes closely linked to those determining the SD antigens. The mutation in H(z1) could have affected both MLC and CML. The AQR–B10.T(6R) and B10.A-(4R)–B10.A(2R) combination would not have an LD target difference. It will require further experimentation to determine whether the SD antigens are in most cases acting as markers to linked LD target loci or are themselves a target for CML.

An intriguing possibility is that the complexity of the genetic control for these reactions (the existence of either more than one type of LD locus or the dual control by an LD-activating locus and an SD target locus) might be paralleled by a dual cell system involved in the reactions. One population of lymphocytes would recognize "LD-activating" differences of the MHC. The proliferation of these cells may be a necessary event allowing the subsequent activation of a second population that would recognize either the SD antigens or the phenotypic product of a closely linked LD target locus (18).

Note Added in Proof.—We have recently demonstrated two separate cell populations' response in MLC and CML reactions [Bach, F. H., M. Segall, K. S. Zier, P. M. Sondel, B. J. Alter, and M. L. Bach. 1973. Cell mediated immunity: separation of cells involved in recognitive and destructive phases. *Science (Wash. D.C.)*. In press.]. In addition, we have shown that if a given responding cell is stimulated by two different cell populations, one differing from the responding cell by LD and the other differing from the responding cell by SD, the responding cell will subsequently be cytotoxic in CML against the target cell carrying the SD antigens (Schendel, D. J., unpublished observations).

SUMMARY

The cell-mediated lympholytic capability of mouse spleen cells stimulated in mixed lymphocyte culture is related to the major histocompatibility complex genotype on target lymphocytes. The strain combinations AQR–B10.T(6R) and B10.A(4R)–B10.A(2R) that result in significant mixed lymphocyte culture activation do not mediate cell-mediated lympholysis on sensitizing target lymphocytes; serologically defined regions (*H-2K* and *H-2D*) are identical within each combination. *H-2K* or *H-2D* region disparity alone does not cause cell-mediated lympholysis. However after mixed lymphocyte culture activation as seen with B10.A–B10.T(6R), a target cell bearing only an *H-2K* region difference from the effector cell is sensitive to cell-mediated lympholysis. Likewise an *H-2D* region difference is an adequate target after mixed lymphocyte culture activation of the effector cell in the combination B10.A(2R)–B10.D2.

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