Brief Definitive Report

ANTIGEN-REACTIVE T CELL CLONES RESTRICTED BY MIXED ISOTYPE A^d_β/E^d_α CLASS II MOLECULES

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The recognition of foreign antigen by $CD4^+$ T lymphocytes is known to be restricted by heterodimeric cell surface glycoproteins consisting of α and β chains called class II (Ia) molecules (reviewed in reference 1). There exist two isotypically related murine class II molecules, namely I-A and I-E. Thus, I-A molecules consist of $A\beta$ and A_α chains, while I-E molecules consist of $E\beta$ and E_α chains. The individual T lymphocyte clone recognizes antigen in the context of I-A or I-E molecules. In F_1 heterozygous mice, not only *cis*-located, but also certain allelic combinations of *trans*-located α and β chains within each isotype freely associate to form I-A and I-E molecules and are expressed on the cell surface to function as restriction elements (2, 3). Such F_1-specific restriction elements are assumed to expand T cell specificity repertoire in F_1 heterozygotes.

Recent serological and biochemical studies suggest the existence of mixed isotype class II molecules ($A\beta E_\alpha$ in mice [4–9] and $DR_\alpha DQ\beta$ in humans [10, 11]). However, T lymphocyte clones that use such mixed isotype class II molecules as restriction elements have not been reported. Our previous studies suggested the existence of alloreactive T cell clones that recognize A^b_β/E^d_α molecules expressed on E^d_α gene-introduced B57BL/6 ($B6E^d_\alpha$) transgenic mouse spleen cells (12). We speculated that such $A^b_\beta E^d_\alpha$ molecules in $B6E^d_\alpha$ transgenic mice are created by the presence of large amounts of free E^d_α molecules (13) that associate with endogenous A^b_β molecules by overcoming low pairing efficiency. In this report, we describe the existence of keyhole limpet hemocyanin (KLH)-specific T cell clones derived from (BALB/c × $B6E^d_\alpha$)F_1 mice that recognize A^d_β/E^d_α molecules as restriction elements. The existence of such mixed isotype class II-restricted T cell clones would provide important implications for the expansion of T cell repertoire as well as the induction of autoimmune phenomenon.

Materials and Methods

Mice. The original E^d_α gene-introduced C57BL/6 ($B6E^d_\alpha$) transgenic mice (14) were obtained from Dr. T. Kishimoto (Osaka University, Osaka, Japan) and bred to homozygous for E^d_α transgenes in our animal breeding facilities. BALB/c, C57BL/6 (B6), and (BALB/c

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× B6)F_1 (CBF_1) mice were purchased from Japan SLC Inc. (Shizuoka, Japan). (BALB/c × B6E^d_α)F_1 mice were made in our animal breeding facilities.

Antigen-specific T Cell Clones. KLH was purchased from Calbiochem-Behring Corp. (La Jolla, CA). Derivation of KLH-reactive T cell clones was performed essentially as described previously (3). Cloning was performed by limiting dilution at a cell density of 3 cells/well, which was repeated several times. 10^4 T cell clones were cultured with 50 μg/ml of KLH in the presence of irradiated (3,300 rad) spleen cells as APCs for 2 d. Proliferative responses of T cell clones were measured by the uptake of [^{3}H]TdR and expressed as a mean cpm of triplicate cultures ± SD.

mAbs. The origin and specificities of mAbs used in this paper are described in Table II. Culture supernatants of each hybridoma cell line were 10-fold concentrated by 50% ammonium sulfate precipitation, dialyzed against PBS (pH 7.2), and used for mAb blocking experiments.

Results

KLH-specific T Cell Lines and Clones Derived from (BALB/c × B6E^d_α)F_1 Mice. KLH-reactive T cell lines and clones were obtained from (BALB/c × B6E^d_α)F_1 mice. The proliferative responses against KLH in the presence of (BALB/c × B6E^d_α)F_1 or CBF_1 APCs are shown in Table I. Almost all the clones showed a similar degree of the KLH-specific proliferative responses in the presence of (BALB/c × B6E^d_α)F_1 and CBF_1 APCs as represented by clones KTF1.1 and KTF2.1. Clones KTF1.2 and KTF2.7, however, showed peculiar restriction specificities. Thus, these clones responded to KLH in the presence of (BALB/c × B6E^d_α)F_1 APCs but not in the presence of CBF_1 APCs. The restriction elements for these clones are not explainable by the conventional knowledge of H-2 genetics since the specificity of class II molecules expressed on (BALB/c × B6E^d_α)F_1 and CBF_1 APCs should theoretically be the same (i.e., $A^b_\beta A^b_\alpha$, $A^d_\beta A^d_\alpha$, $E^b_\beta E^d_\alpha$, $E^d_\beta E^d_\alpha$, $A^b_\beta A^d_\alpha$, and $A^d_\beta A^b_\alpha$). The frequency of clones that showed such peculiar restriction specificity was ~10% from our limiting dilution cloning.

mAb Blocking Experiments of Clone KTF2.7. Since the only difference between (BALB/c × B6E^d_α)F_1 and CBF_1 mice is the presence or absence of E^d_α transgenes,

TABLE I

KLH-specific T Cell Lines and Clones Derived from (BALB/c × B6E^d_α)F_1 Mice

T cell lines and clones	APCs and antigen			
	(BALB/c × B6E^d_α)F_1		CBF_1	
	-	KLH	-	KLH
KTF1	564 ± 89*	4,724 ± 1,163	1,039 ± 189	6,195 ± 933
KTF1.1	113 ± 23	6,681 ± 1,114	169 ± 22	7,359 ± 897
KTF2.1	304 ± 33	9,204 ± 471	68 ± 28	8,497 ± 122
KTF1.2	230 ± 251	4,717 ± 2,971	150 ± 57	582 ± 173
KTF2.7	587 ± 35	9,349 ± 341	573 ± 17	897 ± 55

10^4 T cell lines or clones were stimulated with 50 μg/ml of KLH in the presence of 10^6 APCs from (BALB/c × B6E^d_α)F_1 or CBF_1 mice. Cells were cultured for 2 d. 0.5 μCi of [^{3}H]TdR was added 16 h before the termination of culture. Proliferative responses were assayed by the standard scintillation counting. Results are expressed by the mean cpm of the triplicate cultures ± SD.

* [^{3}H]TdR uptake.

it is possible that such E^d_α transgene products associate with endogenous A^d_β or A^b_β gene products to form mixed isotype $A^b_\beta E^d_\alpha$ or $A^d_\beta E^d_\alpha$ molecules that are expressed on (BALB/c × B6E^d_α)F_1 but not on CBF_1 spleen cells. One of the possible explanations for the restriction specificity of clones KTF1.2 and KTF2.7 would be that they use such mixed isotype $A^b_\beta E^d_\alpha$ or $A^d_\beta E^d_\alpha$ molecules as restriction elements. To demonstrate this, we performed mAb blocking experiments using a large panel of chain-specific anti-class II mAbs. As shown in Table II, the proliferative response of clone KTF2.7 was blocked by both anti-A^d_β and anti-E_α mAbs. None of mAbs having specificities against A_α or E_β could block the clone KTF2.7. Furthermore, anti-I-A mAbs having specificities other than A^d_β could not block the clone KTF2.7. These mAbs could block the proliferation of appropriate T cell clones at the concentrations used in this experiment (data not shown). This result strongly suggests that clone KTF2.7 uses mixed isotype $A^d_\beta E^d_\alpha$ molecules as restriction elements. Our preliminary experiments that demonstrated the ability of APCs from both A^d_β and E^d_α gene-introduced B6 double-transgenic mice to present KLH to clone KTF2.7 (Mineta, T., M. Matsunaga, K. Seki, J. Miyazaki, M. Uno, K. Yamamura, and M. Kimoto, manuscript in preparation) also support this.

TABLE II

mAb Blocking of $A^d_\beta E^d_\alpha$-restricted Clone KTF2.7

Exp.	mAb (references*)	Specificity‡	Dilution of mAb × 1	× 3	× 9	× 27
			cpm			
1	25-9-17s (16,17)	$A^{b,d}_\beta$	215§	3,573	4,589	5,299
	MK-D6 (18,19)	A^d_β	722	3,827	6,661	8,305
	34-5-3 (17,20)	$A^{b,d}_\beta$	653	3,442	5,406	6,695
	Y17 (21)	$E^b_\beta E_\alpha$	8,171	8,119	8,055	7,601
	17-3-3 (22)	E^b_β	8,324	9,438	8,619	8,925
	13/4 (23)	E_α	359	2,505	5,996	7,462
2	3JP (17,24)	A^b_α	7,151	6,978	7,129	7,225
	3F-12 (24)	A^b_α	7,380	7,483	7,032	7,163
	25-5-16 (16)	A^b	7,395	6,106	7,555	7,303
	17/227 (6,23)	$A^{b,d}_\beta$	876	2,060	3,642	3,835
	ISCR-3 (25)	E_α	635	1,031	2,679	3,154
3	K24-199 (26,27)	A^d_α	5,084	5,152	4,832	5,188
	28-16-8 (16,17)	$A^{b,d}_\beta$	530	815	948	1,148
	25-9-3 (16)	A^b	5,155	5,125	5,074	5,711
	34-1-4 (20)	E^d	5,086	4,998	4,603	5,157

10^4 cells of clone KTF2.7 were stimulated with 50 μg/ml of KLH in the presence of APCs from (BALB/c × B6E^d_α)F_1 mice. 20 μl of varying dilutions of mAbs (10-fold concentrated from culture supernatants by 50% ammonium sulfate precipitation) were added at the initiation of culture. Proliferative responses were assayed as described in Table I. The SD of each mean is within 15%. Proliferative responses in the absence and presence of KLH are 587 and 8,810 cpm (Exp. 1), 964 and 7,832 cpm (Exp. 2), and 199 and 5,404 cpm (Exp. 3), respectively.

* References with the original descriptions and the assignment of chain specificities of mAbs.

‡ Specificities only related to this experiment are shown.

§ [^{3}H]TdR uptake.

Discussion

The existence of mixed isotype class II molecules was demonstrated in L cell transfectants (5–8) and in murine and human B lymphoblastoid cell lines (9–11). The possible existence of mixed isotype class II molecules was also reported in several strains of mice (4, 12). Some of these studies suggested that pairing efficiency of isotype-mismatched α and β chains is low compared with isotype-matched α-β pairing (7, 8). This could be the reason why the mixed isotype class II molecule is difficult to detect under the normal situation where the full set of α and β chains exist. Expression of isotype-mismatched α-β pairing seems to occur under the situation where one of the isotype-matched α or β chains does not exist (5–9) or the expression of the isotype-matched α and β chains is unbalanced (10, 11). Transfection experiments of antisense mRNA by Lotteau et al. (11) clearly demonstrated that this is the case. The results in this paper also support this because $B6E^d_\alpha$ transgenic mice contain 20–40-fold more E^d_α transgene products per cell than normal mice (13). The frequency of mixed isotype-restricted T cell clones was estimated to be ~10% from our limiting dilution cloning. Also, mixed isotype-restricted clones sometimes showed poor proliferative responses against appropriate stimulus (12, and this paper). These would reflect the small amount of such mixed isotype $A^d_\beta E^d_\alpha$ molecules expressed on (BALB/c × $B6E^d_\alpha$)F_1 mice.

The significance of such mixed isotype class II molecules in relation to the expansion of the T cell specificity repertoire, as well as the association of disease susceptibility to HLA haplotypes, has already been pointed out by others (15). It would be possible that certain viral infections could induce unbalanced expression of isotype-matched α and β chains, which in turn result in the formation and expression of a certain allelic combination of isotype-mismatched α-β-paired molecules. This could occur not only on the APCs but also on cells that usually do not express class II molecules. Such molecules might be recognized as "non-self" and would be the target for T cell–mediated autoimmune attack. Alternatively, mixed isotype class II molecules may have high affinity for self antigens and T cells with specificity for self antigens, plus newly expressed class II molecules induce or amplify the organ-specific autoimmunity. The situation would be more exaggerated under the local release of lymphokines such as IFN-γ, IL-4, and TNF from activated T cells.

Summary

Mixed isotype $A^d_\beta E^d_\alpha$ class II molecule–restricted antigen-reactive T cell clones were obtained from (BALB/c × $B6E^d_\alpha$)F_1 mice. These T cell clones responded to keyhole limpet hemocyanin in the presence of (BALB/c × $B6E^d_\alpha$)F_1 but not CBF_1 APCs. Both anti-A^d_β and anti-E_α mAbs blocked the proliferative responses of these clones. The frequency of such mixed isotype $A_\beta E_\alpha$-restricted T cell clones in (BALB/c × $B6E^d_\alpha$)F_1 mice was estimated to be ~10% from our limiting dilution cloning. The existence of such mixed isotype class II molecule–restricted T cells would have important implications for the expansion of the T cell repertoire as well as the induction of autoimmunity.

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References

1. Schwartz, R. H. 1986. Immune response (Ir) genes of the murine major histocompatibility complex. *Adv. Immunol.* 38:31.
2. Silver, J., S. L. Swain, and J. J. Hubert. 1980. Small subunit of I-A subregion determines the allospecificity recognized by a monoclonal antibody. *Nature (Lond.).* 286:272.
3. Kimoto, M., and C. G. Fathman. 1980. Antigen-reactive T cell clones. I. Transcomplementing hybrid I-A region gene products function effectively in antigen presentation. *J. Exp. Med.* 152:759.
4. Maloy, W. L., D. H. Sachs, and J. E. Coligan. 1986. Evidence for the association of I-A and I-E molecules in d-haplotype mice. *Mol. Immunol.* 23:263.
5. Germain, R. N., and H. Quill. 1986. Unexpected expression of a unique mixed-isotype class II MHC molecule by transfected L-cells. *Nature (Lond.).* 320:72.
6. Malissen, B., N. Shastri, M. Pierrres, and L. Hood. 1986. Cotransfer of the E_α^d and A_β^d genes into L cells results in the surface expression of a functional mixed-isotype Ia molecule. *Proc. Natl. Acad. Sci. USA.* 83:3958.
7. Sant, A. J., N. S. Braunstein, and R. N. Germain. 1987. Predominant role of aminoterminal sequences in dictating efficiency of class II major histocompatibility complex $\alpha\beta$ dimer expression. *Proc. Natl. Acad. Sci. USA.* 84:8065.
8. Sant, A. J., and R. N. Germain. 1989. Intracellular competition for component chains determines class II MHC cell surface phenotype. *Cell.* 57:797.
9. Spencer, J. S., and R. T. Kubo. 1989. Mixed isotype class II antigen expression. A novel class II molecule is expressed on a murine B cell lymphoma. *J. Exp. Med.* 169:625.
10. Lotteau, V., L. Teyton, D. Berroughs, and D. Charron. 1987. A novel HLA class II molecule (DRα-DQβ) created by mismatched isotype pairing. *Nature (Lond.).* 329:339.
11. Lotteau, V., J. Sands, L. Teyton, P. Turmel, D. Charron, and J. Strominger. 1989. Modulation of HLA class II antigen expression by transfection of sense and antisense DRα cDNA. *J. Exp. Med.* 169:351.
12. Kimoto, M., K. Seki, M. Matsunaga, and T. Mineta. 1989. Unique mixed lymphocyte-stimulating determinants in E_α^d gene-introduced C57BL/6 transgenic mice. *Immunology.* 67:154.
13. Kashiwamura, S.-I., Y. Sadakane, H. Kikutani, T. Kishimoto, and M. Kimoto. 1988. Quantitative analysis of integrated E_α^d gene expression in C57BL/6 transgenic mice. *Immunology.* 65:589.
14. Yamamura, K., H. Kikutani, V. Folsom, L. K. Clayton, M. Kimoto, S. Akira, S. Kashiwamura, S. Tonegawa, and T. Kishimoto. 1985. Functional expression of a microinjected E_α^d gene in C57BL/6 transgenic mice. *Nature (Lond.).* 316:67.
15. Lechler, R. I. 1988. MHC class II molecular structure. Permitted pairs? *Immunol. Today.* 9:76.
16. Ozato, K., and D. H. Sachs. 1981. Monoclonal antibodies to mouse MHC antigens. III. Hybridoma antibodies reacting to antigens of the H-2^b haplotype reveal genetic control of isotype expression. *J. Immunol.* 126:317.
17. Landais, D., B. N. Beck, J.-M. Buerstedde, S. Degraw, D. Klein, N. Koch, D. Murphy, M. Pierres, T. Tada, K. Yamamoto, C. Benoist, and D. Mathis. 1986. The assignment of chain specificities for anti-Ia monoclonal antibodies using L cell transfectants. *J. Immunol.* 137:3002.
18. Kappler, J., B. Skidmore, J. White, and P. Marrack. 1981. Antigen-inducible, H-2-restricted, interleukin 2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition. *J. Exp. Med.* 153:1198.
19. Braunstein, N. S., and R. N. Germain. 1987. Allele-specific control of Ia molecule surface expression and conformation: Implications for a general model of Ia structure-function relationships. *Proc. Natl. Acad. Sci. USA.* 84:2921.
20. Ozato, K., N. Mayer, and D. Sachs. 1981. Monoclonal antibodies to mouse major histocom-

patibility complex antigens. IV. A series of hybridoma clones producing anti-H-2^d antibodies and an examination of expression of H-2^d antigens on the surface of these cells. *Transplantation (Baltimore).* 34:113.

21. Lerner, E. A., L. A. Matis, C. A. Janeway, P. P. Jones, R. H. Schwartz, and D. B. Murphy. 1980. Monoclonal antibody against an Ir gene product? *J. Exp. Med.* 152:1085.
22. Ozato, K., N. Mayer, and D. H. Sachs. 1980. Hybridoma cell lines secreting monoclonal antibodies to mouse H-2 and Ia antigens. *J. Immunol.* 124:533.
23. Hammerling, G. J., U. Hammerling, and H. Lemke. 1979. Isolation of twelve monoclonal antibodies against Ia and H-2 antigens. Serological characterization and reactivity with B and T lymphocytes. *Immunogenetics.* 8:433.
24. Beck, B. N., J.-M. Buerstedde, C. J. Krco, A. E. Nilson, C. G. Chase, and D. J. Mckean. 1986. Characterization of cell lines expressing mutant I-A^b and I-A^k molecules allows the definition of distinct serologic epitopes on Aα and Aβ polypeptides. *J. Immunol.* 136:2953.
25. Watanabe, M., T. Suzuki, M. Taniguchi, and N. Shinohara. 1983. Monoclonal anti-Ia antibodies crossreactive with the Ia-homologues of the mammalian species including humans. *Transplantation (Baltimore).* 36:712.
26. Koch, N., G. Hammerling, N. Tada, S. Kimura, and U. Hammerling. 1982. Cross-blocking studies with monoclonal antibodies against I-A molecules of haplotypes b, d, and k. *Eur. J. Immunol.* 12:909.
27. Buerstedde, J.-M., L. R. Pease, M. P. Bell, A. E. Nilson, B. Buersteddes, D. Murphy, and D. J. Mckean. 1988. Identification of an immunodominant region on the I-A β chain using site-directed mutagenesis and DNA-mediated gene transfer. *J. Exp. Med.* 167:473.