Cross Reactivity of T Cell Receptor on Memory CD8+ Cells Isolated after Immunization with Allogeneic Tumor Cells

E. L. Pobezinskaya, L. A. Pobezinskii, Yu. Yu. Silaeva, T. V. Anfalova, L. M. Khromykh, T. S. Tereshchenko, E. S. Zvezdova, and D. B. Kazanskii

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Experiments on mice deficient in expression of class I major histocompatibility complex molecules showed that memory CD8+ cells recognizing the alloantigen by the direct allogeneic recognition mechanism selectively proliferated in response to heated allogeneic cells. Adoptive transfer of memory cells from mice expressing green fluorescent protein transgene to wild-type animals showed for the first time that long-living memory cells suppress the response of naive T cells and abolish their involvement in the pool of memory cells. The pool of long-living memory T cells was obtained *in vitro* with heated allogeneic stimulators. Apart from immunizing alloantigen, this clone recognized foreign molecules of the major histocompatibility complex. Cloning and sequencing of rearranged regions in memory T cells showed that two α-chains and one functional β-chain are rearranged in cells of this pool. Only one α-chain was capable of forming protein product, which determines expression of only one form of T cell receptor. Experimental data directly confirm the hypothesis about degeneracy of recognition of allelic products of major histocompatibility complex molecules by T cell receptors. Suppression of the response of naive cells by memory cells probably underlies a previously unknown type of polarization of the immune response and determines clonal dominance and peripheral selection of T lymphocytes.

Key Words: *T lymphocyte; memory cell; proliferation; cloning; alloantigen*

Most recent studies of memory cells were performed on experimental models of transgenic T cell receptors (TCR), which makes impossible to determine the role of selection and antigen-specific recognition in biological activity of T cells belonging to this subclass [2,4]. An alternative approach is to study allogeneic response in animals congenic by the major histocompatibility complex (MHC). This method allows evaluation of the type of ligands recognized by T cells. The absence of reliable phenotypic and functional criteria for identification of memory cells is a serious problem in these studies [8]. Our previous experiments showed that TCR, but not naive T cells, are able to respond *in vitro* to an antigen presented on the surface of cultured functionally inactive antigen-presenting cells [10,11].

In the present study, memory cells were stimulated with the H-2K^b alloantigen. This alloantigen is present on the surface of antigen-presenting cells subjected to acute heat shock. The specific goal was to evaluate selectivity of this stimulation, to determine the phenotype of responding T cells, to study the mechanism for recognition of the alloantigen, and to isolate the pool of memory T cells.

N. N. Blokhin Russian Cancer Research Center, Russian Academy of Medical Sciences, Moscow. ***Address for correspondence:*** kazansky@dataforce.net. D. B. Kazanskii

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MATERIALS AND METHODS

Experiments were performed on inbred mice obtained from the Department for Breeding of the N. N. Blokhin Russian Cancer Research Center (Table 1). β_2-Microglobulin knockout mice (C57BL/6J-B2m^{tm1Unc}), Tap-1 molecule knockout mice (C57BL/6J-Tap1^{tm1Arp}), and transgenic C57BL/6-TgN(ACTbEGFP)1Osb mice were obtained from the Jackson Laboratory (Bar Harbor, Main, USA) and bred at the Laboratory for Mechanisms of Immune Regulation. Genotyping was performed according to recommendations presented at the Jackson Laboratory web site.

For breeding B10.D2(R101) mice expressing green fluorescent protein (GFP) transgene, C57BL/6-TgN (ACTbEGFP)1Osb mice were crossed with B10.D2 (R101) mice and first-generation hybrids were backcrossed with animals of the parent strain. Survived second-generation B10.D2(R101) homozygotes expressing the transgene were selected after intraperitoneal injection of 2×10^7 thymoma EL4 (K^bD^b) cells. Ascitic thymoma was passed in syngeneic C57BL/6 mice. The transgene was detected by green fluorescence of open body parts in animals using an UV-transilluminator.

After 2 months, splenocytes from immunized animals were used as a source of long-living memory cells not exhibiting cytotoxic activity *ex vivo*. For evaluation of the effect of long-living memory cells on the response of naive cells thymoma cells and 2.5×10^7 splenocytes from immunized or intact B10.D2(R101) mice expressing the GFP antigen were administered to B10.D2(R101) mice.

Stimulating (stimulators) and responding lymphocytes (responders) were delicately squeezed from the stroma of mouse spleen using a Potter homogenizer with a conical pestle. Erythrocytes were removed by hypotonic shock. Mononuclear cells were washed by centrifugation at 200*g* for 7 min. Viable cells were counted in a mixture of trypan blue and eosin. Stimulating splenocytes were γ-irradiated on a ^{137}Cs source (25 Gy) or treated with mitomycin C in a dose of 25 μg/ml at 37°C for 30 min. Acute heat shock was induced by incubation at 45°C for 60 min.

For mixed lymphocyte culture (MLC) reaction the responders from B10.D2(R101) mice (3×10^6 cells) were incubated with irradiated or heated allogeneic and syngeneic stimulators (ratio 1:1) in complete RPMI-1640 medium containing 5% human serum, 0.016% gentamicin sulfate, 4 mmol L-glutamine, 20 mmol HEPES, and 5×10^{-5} M 2-mercaptoethanol at 37°C, 5% CO_2, and absolute humidity. After 3-day incubation, the cell cultures were studied by the method of flow cytofluorometry and the percent of blast $CD8^+$ cells not expressing GFP was determined.

The intensity of proliferation was determined by incorporation of ^{3}H-thymidine over 18 h. Memory cells were enriched in primary MLC with heated allogeneic stimulators for 10 days, collected, purified from dead cells by centrifugation in a Ficoll-Verografin density gradient (1.09), and cloned by the limiting-dilution method in 96-well flat-bottom plates containing the fraction of allogeneic irradiated adherent splenocytes and 10 U/ml mouse interleukin-2. Growing clones were restimulated at 8-10-day intervals. EL4 mice (kindly provided by Prof. A. V. Chervonskii from the Jackson Laboratory, Bar Harbor, Main, USA) with high-intensity production of interleukin-2 were used as the source of this cytokine. Interleukin-2 concentration was determined by the intensity of MTT catabolism in interleukin-2-dependent CTLL-2 cells [12].

TABLE 1. Haplotypes of MHC in Experimental Mice

Strain	Expression of allelic products in H-2 complex				
	K	I-A	I-E	D	L
B10.D2(R101)	d	d	d	b	—
B10.A	k	k	k	d	D
B10.A(4R)	k	k	k/b	b	—
B10.A(3R)	b	b	b/k	d	d
B10.MBR	b	k	k	q	q
B10.AKM	k	k	k	q	q
B10.D2	d	d	d	d	d
B10.M	f	f	—	f	—
C57BL/10	b	b	—	b	—
C57BL/6	b	b	—	b	—
C3H	k	k	k	k	—

Note. — no expression.

The composition of cells populations was estimated by flow cytofluorometry with phycoerythrin-labeled anti-CD4 and allophycocyanin-labeled anti-CD8α antibodies (Pharmingen). The cells were stained with antibodies according to recommendations of the manufacturer. Dead cells stained with propidium iodide were excluded from the analysis. Samples were examined on a FacsCalibur flow cytofluorometer. The results were analyzed using WinMDI 2.8 software.

We studied expression of TCR variants. Total RNA was extracted from cultured cells with Tri Reagent (Sigma) according to recommendations of the manufacturer. Isolated RNA was dissolved in deionized water. The structure of primers and conditions for reverse transcription polymerase chain reaction (PCR) were described elsewhere [5]. After PCR the reaction mixture was separated by electrophoresis in 2% agarose gel with ethidium bromide (Tris-borate buffer, 8 V/cm for 45 min). The gel was photographed and studied on an UV-transilluminator equipped with a CD-camera. Cloning of amplified products into the TA vector was followed by isolation of plasmid DNA and sequencing (Genom Center, V. A. Engelhardt Institute of Molecular Biology). The results of sequencing were analyzed on-line by means of Chromas v. 1.45, DNAssist v. 1.0, and BLAST softwares.

For each experimental group the arithmetic mean and error were calculated. The data were processed using Student's *t* test.

RESULTS

In *in vitro* MLC $CD8^+$ cells from intact animals do not proliferate in response to allogeneic stimulators subjected to acute heat shock (Fig. 1). By contrast, heated allogeneic stimulators selectively increased the ratio of

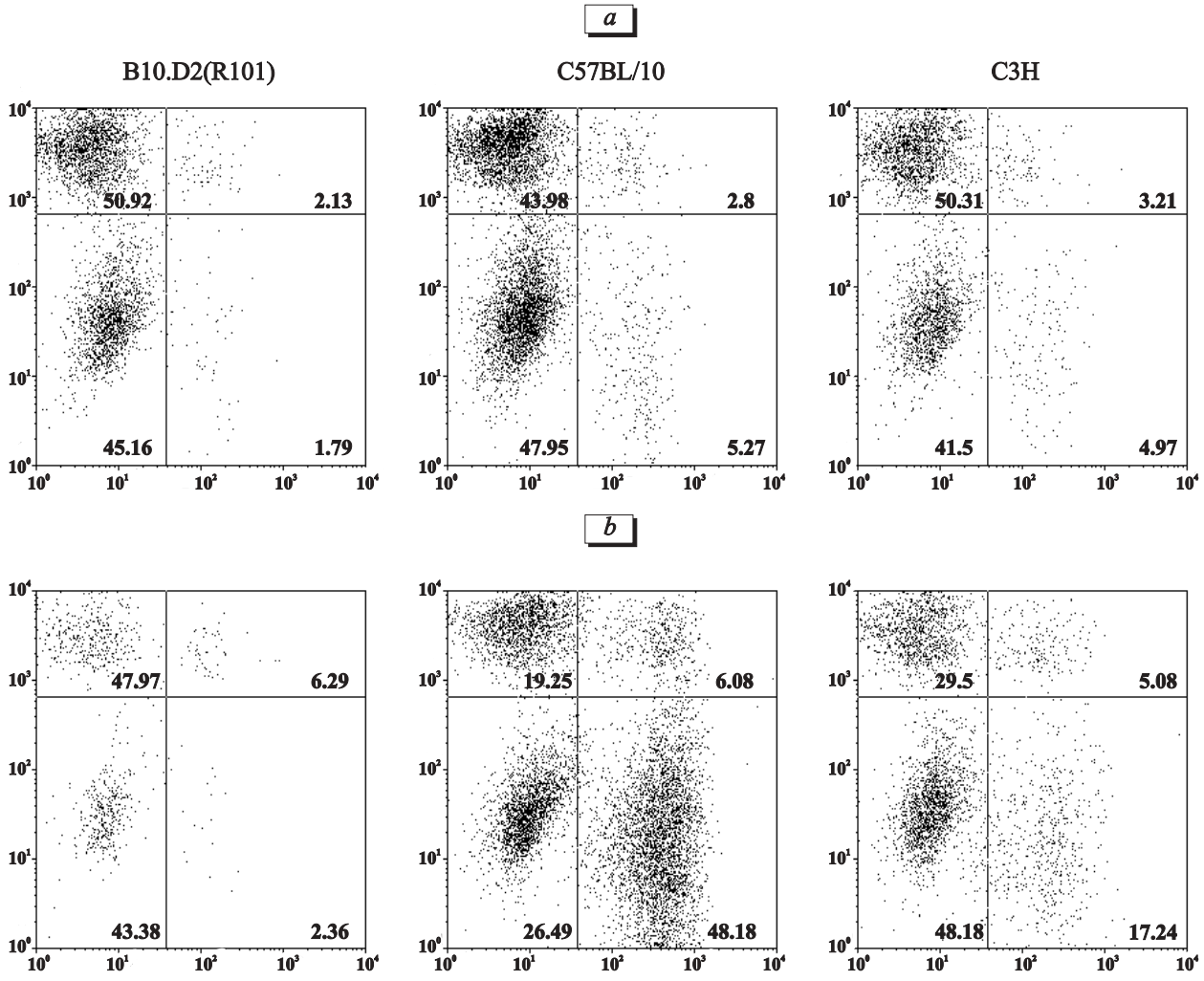

Fig. 1. Blast population of splenocytes from intact (*a*) and immunized B10.D2(R101) mice (*b*) after incubation in the mixed lymphocyte culture with syngeneic (R101), allogeneic (C57BL/10), and foreign stimulators (C3H) subjected to heat shock. Abscissa: staining with antibodies against CD8. Ordinate: staining with antibodies against CD4.

TABLE 2. Effect of Adoptive Transfer of GFP$^+$ Memory Cells on Recruitment of Naive GFP$^-$ Cells into the Pool of Memory CD8$^+$ Cells in the Recipient

Administered to recipients			Stimulators in MLC	CD8$^+$GFP$^-$ T cells in blast population, %
GFP$^+$ splenocytes from intact animals	GFP$^+$ splenocytes from immunized animals	thymoma EL4 cells		
+	—	+	B10.D2(R101), mitomycin C	3.93±0.97
			C57BL/10, mitomycin C	43.3±16.0^o
+	—	+	B10.D2(R101), heated	2.38±0.04
			C57BL/10, heated	32.6±6.81^o
—	+	+	B10.D2(R101), mitomycin C	3.24±0.08
			C57BL/10, mitomycin C	8.06±0.77*
—	+	+	B10.D2(R101), heated	2.88±0.23
			C57BL/10, heated	8.51±0.55*
—	+	—	B10.D2(R101), mitomycin C	3.73±0.52
			C57BL/10, mitomycin C	7.23±1.94
—	+	—	B10.D2(R101), heated	3.02±0.68
			C57BL/10, heated	3.65±0.32

Note. *$p<0.001$: significant suppression of the response of CD8$^+$GFP$^-$ cells in a recipient by memory GFP$^+$ cells of a donor, compared to the response of recipients injected with GFP$^+$ splenocytes from nonimmunized mice (^{o}).

blast CD8$^+$ cells in splenocyte culture from B10.D2 (R101; $K^dI^dD^b$) mice immunized with thymoma EL4 (K^bD^b) cells. This reaction is specific to the alloantigen, since only a small number of T cells responded to heated foreign stimulators from C3H (H-2^k) mice.

It was interesting to evaluate whether naive T lymphocytes are involved in memory cell response. Adoptive transfer of transgenic memory cells during immunization blocked the proliferative response of memory CD8$^+$ cells in a recipient to heated and mitomycin C-treated allogeneic stimulators (Table 2).

For evaluation of the mechanism of recognition of the H-2K^b antigen by memory cells we studied the response of cells to heated and mitomycin C-treated stimulators from C57BL/6 mice, C57BL/6J-B2m^{tm1Unc} β_2-microglobulin knockout mice, and C57BL/6J-Tap1^{tm1Arp} Tap-1 molecule (transport-associated protein) knockout mice in the MLC test. Both knockout strains were characterized by normal synthesis of heavy chains of H-2K^b antigen and their proteosome degradation and impaired assembly and expression of MHC—β_2-microglobulin—peptide complex on cell membrane [1,7]. Therefore, the direct allogeneic recognition of the complex on the surface of heated cells from knockout mice is impaired, while indirect allogeneic recognition of alloantigen fragments in the context of MHC molecules in responding dendrite cells (cross-priming) is preserved [3]. The response of memory cells to heated stimulators from knockout animals was significantly suppressed, therefore the major mechanism of alloantigen recognition by memory cells was direct allogeneic recognition (Table 3).

These results created the possibility for selective isolation of allospecific memory T cells capable of directly recognizing allogeneic MHC molecules. Memory cells were stimulated with heated allogeneic stimulators in MLC and cloned by the method of maxi-

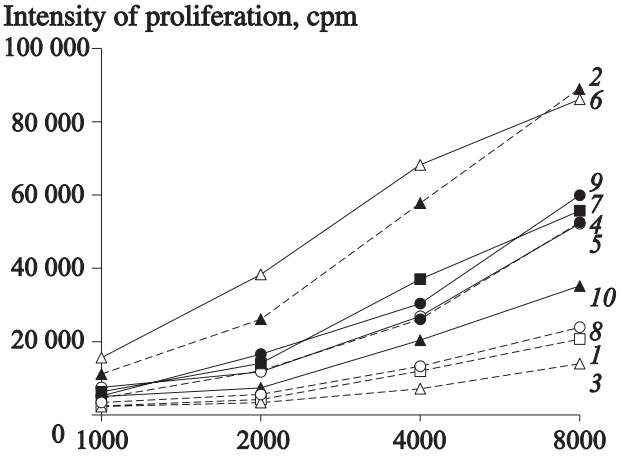

Fig. 2. Incorporation of ^{3}H-thymidine in the clone of MCC-1 cells stimulated with irradiated splenocytes from mice of various strains in the presence of 10 U/ml mouse interleukin-2. Abscissa: count of responding cells per well. *1* — B10D2(R101), *2* — B10.A, *3* — B10.A(4R), *4* — B10.A(3R), *5* — B10.MBR, *6* — B10.AKM, *7* — B10.D2, *8* — B10.M, *9* — C57BL/10, *10* — C3H.

	V-segment		N	J-segment
Nonproductive rearrangement	TCRAV5-TTCTGCGCAG]		CCA	[TGTCTAATTA-J-alpha_14-4
	V-segment			J-segment
Productive rearrangement	TCRAV13S1-TGTGCTGCAA]			[CAGGCACTGG-J-alpha_new08
	V-segment	D-segment	N	J-segment
Productive rearrangement	TCRBV3S1-AGCAGTCTGT]	[GGACAGGGGGC]	TGG	[GAACAGTACT-J2S7

Fig. 3. Sequences of rearranged regions in subunits of T cell receptors of the MCC-1 clone. Square brackets: boundaries of V- and J-segments in α-chains and V-, D-, and J-segments in β-chains. Underlying: N-nucleotides.

mum dilution. Only 1 of 12 clones (MCC-1) had a long survival time and could be maintained *in vitro* for a long time. The MCC-1 clone expressed TCRα/β and CD8 coreceptor (data not shown). This clone exhibited practically the same proliferative response to H-2K^b-expressing stimulators C57BL/10 (K^{b}I^{b}D^b), B10.A(3R) (K^{b}I-A^{b}I-E$^{b/k}$D^{d}L^d), and B10.MBR (K^{b}I^{k}D^{q}L^q). However, the clone was characterized by cross-reactivity with targets expressing MHC products H-2D^d(L^d) and H-2D^q(L^q) in the absence of immunizing alloantigens (Fig. 2). These products were more potent than the immunizing H-2K^b alloantigen in inducing proliferation of the clone. It was probably related to supraoptimal stimulation of the clone by the immunizing antigen, which resulted in cell death. Reverse transcriptase PCR with primers for variable and constant segments of TCR β-chains, cloning, and sequencing of the rearranged region showed that the β-chain gene consists of the Vβ3 segment (TCRBV3S1) coupled to D1 and J2S7 segments and induces expression of the complete β-chain (Fig. 3). Expression of TCR α-chains was not characterized by allelic exclusion. It can be hypothesized that there are T cells expressing 2 α-chains and, therefore, 2 TCR [6]. Typing of variable fragments in TCR α-chains of the MCC-1 clone by reverse transcriptase PCR with primers specific to variable segments of α-chains and primer for the constant region of the α-chain resulted in amplification of products with primers for Vα5, Vα13, Vα14, Vα17, and Vα19. Sequencing of these products showed that the sequence of transcript DNA amplified by primers for Vα17 is not typical of TCR. The sequence of transcript DNA amplified by the primer for Vα5 is a product of completely rearranged α-chain (TCRAV5-J-alpha_14-4) not translating the protein product due to the presence of stop-codons. Sequencing of products amplified by primers for Vα13, Vα14, and Vα19 identified the same sequence of the Vα13 gene (TCRAV13S1) coupled to the J-alpha_new08 sequence and expressing the complete α-chain. Identification of 2 rearranged genes for TCR α-chains in the MCC-1 clone (1 nonfunctional chain) showed that our analysis was sufficient. The clone expressed only one TCR α-chain and, therefore, one form of TCRα/β.

TABLE 3. Proliferative Response of Memory Cells to Stimulators from β_2-Microglobulin and TAP (Transport Associated Protein) Knockout Mice

Stimulators	Intensity of proliferation	
	splenocytes from intact animals	splenocytes from immunized animals
Treatment with mitomycin C		
B10.D2 (R101)	2834±104	4096±274
C57BL/6	25 146±1752	41 279±1719
β_2m KO	14 054±1038	53 726±2009
TAP KO	47 905±4063	44 374±8005
Heating		
B10.D2 (R101)	3859±703	3941±399
B6	3646±259	19 373±3065°
β_2m KO	3209±467	8032±1395*
TAP KO	5138±388	5215±75*

Note. *Significant (p<0.001 suppression of the response compared to response to wild-type allogenic stimulators (°). KO: knokout; β_2m: β_2-microglobulin.

Our results indicate that TCR of memory MCC-1 cells is presented by individual heterodimer. Therefore, the specificity and cross-reactivity of the clone are associated with the individual structure of its receptor. Experimental data directly confirm the hypothesis about degenerative recognition of allelic products in MHC by receptors on T cells (*e.g.*, TCR) [9,13,14]. Memory cells suppress the response of naive cells, which probably underlies a previously unknown type of polarization of the immune response and determines clonal dominance and peripheral selection of T lymphocytes.

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