ISSN 0026-8933, Molecular Biology, 2010, Vol. 44, No. 2, pp. 277–286. © Pleiades Publishing, Inc., 2010.
Original Russian Text © E.S. Zvezdova, Yu.Yu. Silaeva, M.S. Vagida, E.V. Maryukhnich, A.V. Deikin, T.G. Ermolkevich, S.G. Kadulin, E.R. Sadchikova, I.L. Goldman, D.B. Kazansky, 2010, published in Molekulyarnaya Biologiya, 2010, Vol. 44, No. 2, pp. 311–322.

CELL MOLECULAR BIOLOGY

UDC 612.006-092.19:612.017.1-063

Generation of Transgenic Animals Expressing the α and β Chains of the Autoreactive T-Cell Receptor

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Received April 21, 2009
Accepted for publication August 6, 2009

Abstract—Transgenic animal analysis has become a key approach used to study the gene functions and to model various human diseases, including autoimmune disorders. Such disorders are caused by the activation of T-cell clones whose T-cell receptors (TCRs) have a high affinity for syngeneic MHC molecules. The genes coding for the α and β chains of the autoreactive TCR were cloned from hybridoma 7, which was specific for syngeneic A^b MHC class II molecules. Amplified DNA fragments containing rearranged genomic DNA of the α and β chains of hybridoma 7 were cloned into special cassette vectors that contained the natural promoter and enhancer elements ensuring direct expression of the α- and β-chain genes in T cells of transgenic animals. The animals obtained with the vectors expressed the α or β chain on the majority of peripheral T cells. The animals are suitable for studying the features of the intrathymic selection and maturation of T cells and provide an experimental model for developing new approaches to therapy of autoimmune diseases.

DOI: 10.1134/S0026893310020135

Key words: T lymphocyte, cloning, transgene, MHC, TCR

INTRODUCTION

Introduction of foreign genes into cells of experimental animals has recently come to be the main method for studying intricate multicomponent biological processes, such as cell differentiation and the developmental regulation of the expression patterns of tissue-specific genes. One of the relevant techniques was described in the 1980s and is based on a DNA microinjection into the oocyte pronucleus and the subsequent transfer of the injected oocytes into the oviducts of pseudopregnant females. In some cases, the procedure leads to stable integration and expression of foreign DNA in the genome of the resulting progeny. generation of such animals is important for the progress of modern biological sciences, first and foremost, immunology, since the roles of various proteins in the immune system can be studied in vivo via transduction, amplification, or deletion of the corresponding genes.

T cells recognize foreign antigens presented in the form of peptides bound with cognate major histocompatibility complex (MHC) class I and class II molecules. T-cell receptors (TCRs) consist of α and β chains, which are associated with the CD3 protein complex and are responsible for signal transduction within the T cell. The diversity of α and β chains of TCRs results from somatic rearrangements of the V (or D in the case of the β chain) and J segments of the variable TCR gene region by the RAG1 and RAG2 recombinases. During such rearrangements, terminal nucleotides are occasionally removed from the modified segments and/or extra nucleotides are added to the interface between the V (D) and J segments by terminal deoxynucleotidyltransferase (TdT) [1, 2]. Thus, recombination of the V, D, and J segments in a particular cell may yield a large clonal diversity of TCRs (up to 10^{13} variants). The further fate of each clone depends on positive and negative selection within the thymus. Selection preserves clones whose TCRs are capable of interacting with cognate MHC molecules associated with endogenous peptides of the thymic environment (positive selection) and eliminates clones that are capable of high-affinity interactions with cognate MHC/peptide complexes (negative selection) to prevent autoimmune reactions. However, autoreactive T cells escape negative selection in some

Abbreviations: MHC—Major Histocompatibility Complex; MLR (Mixed Lymphocyte Reaction)—reaction of mixed culture of lymphocytes; TCR—T cell receptor; TDT—terminal deoxynucleotide transferase; APC—antigene presenting cell; IL-2—interleukin-2; PBS—phosphate-salt buffer; RAG1 and RAG2 (Recombination Activating Genes)—recombinases realizing restructuring of gene segments of immunoglobulins and TCP; HEPES—4-(2-hydroxyethyl)-1piperazineethanesulfonic acid; KSOM—medium for development and growth of pretransplantation embryos; GFR—Green Fluorescent Protein.

cases, leading to various autoimmune disorders, which occur in more than 5% of the population.

Animal strains that express rearranged TCR transgenes are now the main tool to study the basic processes of T-cell biology. Transgenic TCR chains substantially inhibit the rearrangements of endogenous TCR chains. Thus, the transgenic TCR is expressed in the majority of T cells at least until intrathymic selection, which forms the repertoire of mature T cells. This makes it possible to track the fate and behavior of T cells with the known specificity. Such studies are difficult to perform with wild-type animals, since their T cell populations of interest are usually heterogeneous and small. Most studies in immunology focus now on T-cell development, the choice of a differentiation lineage, and the formation of T-cell specificity to "non-self" and tolerance of "self." One of the topical current problems is to study how autoreactive T cells are selected and what mechanisms underlie autoimmune disorders.

Autoreactive T cells that find their way to the periphery in a minor amount are inactive, which is due to additional peripheral mechanisms inducing tolerance. This circumstance makes it difficult to identify and to study the autoreactive T-cell population. Immune system disorders and activation of autoreactive T cells usually occur with age as a result of chronic infections.

To identify the autoreactive T cells and to determine how they escape negative selection, studies are performed with model animals that are transgenic for costimulatory molecules or are deficient in the AIRE protein, costimulation inhibitors, or regulatory T cells. Such animals often develop autoimmune disorders, but the type of the disorder is usually unpredictable. Several studies have been carried out with genetically modified animals whose cells expose a MHC molecule complex with one invariant peptide on their surface. Negative selection is altered in such animals, while positive selection is preserved, generating a repertoire of diverse T cells [5–7]. For instance, Chervonsky and colleagues [7] have recently constructed a mouse model of psoriasis-like dermatitis. On the one hand, such model animals are convenient to study autoreactive T cells, since their selected T cells are capable of responding to MHC molecules of syngeneic wild-type animals. Another way to characterize such a T cell is to generate animals that express a transgenic autoreactive TCR in order to study and to identify the general regularities of autoreactive T-cell selection. On the other hand, these models are still artificial, since the T-cell repertoire is originally altered to a great extent in such animals.

A protocol has been developed in our lab to construct mouse T-cell hybridomas that express the rearranged genes for autoreactive TCRs of T cells from wild-type mice, which lack any defect in selection during thymocyte differentiation. Such hybridomas offer great advantages over genetically modified model animals, which usually serve as a source of autoreactive T cells [8]. Since allelic exclusion is incomplete in the case of the α chain, a portion of T cells express both alleles of the rearranged α chains, and two functional TCRs are consequently exposed on the cell surface. The functional consequences of this phenomenon are still unclear, although a hypothesis has been advanced that one TCR allows the other one to escape negative selection in the thymus [9].

In this work, we studied the TCR specificity for T cells that expressed two rearranged functional genes for the TCR α chain. Using cassette vectors for targeted expression of transgenic TCRs in mouse T cells, we constructed animals with vertical transmission of a transgene for the α- or β chain of an autoreactive TCR. The transgenes under study were expressed in a substantial portion of peripheral T cells of the resulting animals and were associated with endogenous chains to produce functional TCRs. Our transgenic mouse models are suitable for studying the corresponding autoimmune disorders and analyzing how autoreactive T cells escape negative selection.

EXPERIMENTAL

Animals. Mice of the C57BL/6 ($K^bI\text{-}A^bD^b$), C57BL/6J-H2^{bm12} ($K^bI\text{-}A^{bm12}D^b$), and C57BL/6J-H2^{bm3} ($K^{bm3}I\text{-}A^bD^b$) strains were obtained from the breeding facility of the Blokhin Cancer Research Center. The F_1 hybrid (CBA × C57BL/6) strain was from the Stolbovaya nursery.

Cell lines. The 4G4 and EL4 cell lines (kindly provided by Chervonsky, Department of Pathology, University of Chicago, United States) were grown in vitro by passages in RPMI 1640 (Pan Eko) supplemented with 4 mM L-glutamine and 10% fetal calf serum (Pan Eko). CTLL-2 cells were maintained in the same medium supplemented with 5 MU/ml IL-2. The EL4 cell line with a high spontaneous production of mouse IL-2 was used as a source of this cytokine. EL4 cells were maintained in DMEM supplemented with 4 mM L-glutamine and 10% fetal calf serum. IL-2 was quantified by the MTT catabolism assay with IL-2-dependent CTLL-2 cells with minor modification [10].

Analysis of the V, D, and J segments and complementarity-determining regions (CTRs) of TCR. The amplified TCR gene fragments were cloned in pTZ57RT, and the resulting clones were sequenced at the Genom Center, Engelhardt Institute of Molecular Biology). Nucleotide sequences were analyzed using the Chromas v. 1.45 program. Amino acid sequences were deduced, and stop codons were identified in the rearranged region with the DNAssist v. 1.0 program. The BLAST package was used to identify the V-, D-, and J-segment sequences, template-dependent palindromic (P) nucleotides, and nontemplate-dependent (N) nucleotides added by TdT at the interface of the gene segments. The same package was used to assemble the hypothetical full-length cDNA sequences of

the identified TCR α and β chains with the help of the best characterized genomic sequence of the α/δ- and β loci of the 129 mouse cell line. The full-length cDNAs were cloned in retroviral vectors.

Generation of 4G4 transfectants expressing the TCR α- and β-chain transgenes with coreceptors. The method was described in detail previously [10]. In brief, the cDNAs amplified from hybridomas 7 with primers directed to the full-length TCR chain genes were cloned in retroviral vectors. The pMigRI vector (Invitrogen, United States), which contained the green fluorescent protein (GFP) gene with the internal ribosome entry site (IRES), was used to clone the α-chain cDNAs. The pMINV vector (Invitrogen, United States), which contained the neomycin resistance gene with the IRES, was used to clone the β-chain cDNAs. The resulting constructs were sequenced. The sequences coding for the α and β chains of the CD8 coreceptor, which were kindly provided by D. Littman (Skirball Institute of Biomolecular Medicine), were cloned in pMSCVpuro and pMSCVhygro, respectively. The sequence coding for the CD4 coreceptor was kindly provided by Chervonsky and was cloned in pLenti6/V5-D-TOPO.

To obtain viruses, Hek293 fibroblasts (Invitrogen, United States) were cotransfected with the resulting constructs and accessory plasmids. After infecting 4G4 thymoma cells with the viruses, the medium was replaced by RMPI 1640 supplemented with a selective antibiotic. Since pMigRI lacked an antibiotic resistance gene and coded for GFP, cells expressing TCR α chains were analyzed by flow cytometry and cloned by the limited dilution method. CD4 and CD8 were detected on the surface of the resulting clones by flow cytometry. Clones that displayed stable expression of the coreceptors and an intense staining with the corresponding antibodies were selected for further experiments.

Cell suspensions. Splenocytes were gently squeezed from mouse spleen in a Potter homogenizer with a conic pestle. Viable cells were counted after staining with trypan blue–eosin. To test the 4G4 transfectants, cells were plated in flat-bottom plates at approximately 0.1×10^6 cells per well; the wells contained antigen-presented cells (0.5×10^6 cells per well), which expressed different MHC alleles. The plates were frozen after 1 day, and the supernatants were subsequently tested for the capability of sustaining the growth and viability of CTLL-2 cells [4].

Cloning of the rearranged genomic sequences for the TCR α and β chains from hybridoma 7 in the pTα and pTβ cassette vectors. We used the primers TCRAV11S4orS5SmaIF (5'-ccagcccggggacaggggc-catg-3') and JDK1SacIIR (5'-aggccgcggcacctgtaga-cactt-3') to clone the α-chain DNA sequence and BV8S3XhoIF (5'-acactcgagtgacttggtcgcgag-3') and BJ2S7SacIIR (5'-ttgaccgcggtctcctactatcga-3') to clone the β-chain DNA sequence. The products amplified from hybridoma 7 genomic DNA with primers specific to the V and J segments of the α- and β chains were cloned using a TA cloning kit (Invitrogen, United States) and sequenced (Genom Center, Engelhardt Institute of Molecular Biology). The sequencing results were analyzed using the programs Chromas v. 1.45, DNAssist v. 1.0, and BLAST (on line). Then, the α-chain sequence cloned in the TA vector was digested with *Sma*I and *Sac*II (Fermentas, Lithuania), purified from agarose gel with a QIAGEN kit (United States), and cloned again in pTα linearized with *Sma*I and *Sac*II. The β-chain sequence cloned in the TA vector was digested with *Xho*I and *Sac*II (Fermentas, Lithuania) and recloned in pTβ linearized with *Xho*I and *Sac*II [3]. Prior to injecting DNA, the ampicillin resistance gene was excised from the cassette vectors with *Sal*I in the case of pTα and *Kpn*I in the case of pTβ.

Generation of transgenic animals. To obtain primary transgenic animals, DNA (5 ng/μl in the TE buffer) was injected into the male pronuclei of fertilized ovales of F_1 (CBA × C57BL/6) mice, and the surviving microinjected zygotes were grafted to pseudopregnant recipients.

ovales were obtained by inducing superovulation. For this purpose, young F_1 (CBA × C57BL/6) female mice (12–13 g) were injected intraperitoneally with 8 units of pregnant horse serum gonadotropin (PHSG, MOSAGROGEN) and, after 46 h, 8 units of human chorionic gonadotropin (HHG) Moscow Endocrine Company). The females were placed together with F_1 (CBA × C57BL/6) males. The fact that mating had occurred was verified next morning by the presence of the copulative plug. The superovulation schedule was as follows: PHSG injection at 1:00 p.m., HHG injection after 46 h at 11:00 a.m., and placing with males the same day at 5:00 p.m. Donors were selected next day at 9:30 a.m. The room was illuminated from 7:00 a.m. to 7:00 p.m.

Females with copulative plugs were sacrificed via cervical dislocation and used to isolate the oviducts. ovales were washed from the oviducts with HEPES-KSOM supplemented with hyaluronidase (Sigma) under a Zeiss Stemi DV4 binocular microscope (magnification ×32), using glass capillaries with an inner diameter of approximately 100 μm. The capillaries were made using a Narishige PC-10 puller and a Narichige MF-900 microforge. ovales were cultured in a HEPES-KSOM or KSOM drop covered with mineral oil for 2 h (37°C, 5% CO_2) and then placed into a microinjection cell. Microinjections were performed in HEPES-KSOM under a Zeiss Axiovert 200M microscope (magnification 400×–600×), using Narishige micromanipulators. Microinjection needles were made using a Sutter Instrument Co P-97 puller. After microinjection, surviving cells were transferred into a drop of KSOM or HEPES-KSOM covered with mineral oil and cultured for 1 h to identify viable cells.

To obtain the recipients, adult F_1 (CBA × C57BL/6) females weighing no less than 24 g were

combined with vasectomized males of the same strain. Pseudopregnant recipients were selected next morning by the presence of copulative plugs.

After microinjection, surviving zygotes were transplanted into the left oviduct of a pseudopregnant female. Depending on the number of cells surviving the microinjection, 10–20 zygotes were transplanted to each female. The females were immobilized during surgery with Avertin (a 2.5% aqueous solution of the preparation containing 1 g of 2,2,2-tribromoethanol in 1 ml of 2-methyl-2-butanol). Avertin was injected intraperitoneally at 15 μl per gram body weight.

Progeny and its testing for the transgene. If delivery did not occur 21 days after grafting microinjected ovales, the recipient was sacrificed via cervical dislocation, cesarean section was performed, and the pups were fed by foster mothers. Tail tips of the mice resulting from microinjections were amputated 14–21 days after birth, and DNAs were isolated and tested for the transgene by PCR. DNA isolation followed a standard protocol (Molecular Cloning, United States). Amplification with Taq DNA polymerase (GIBCO/BRL, United States) was performed in the buffer (containing 2.5 mM $MgCl_2$) supplied with the enzyme in the presence of 2 mM dNTP and 10 pmol of each primer and included denaturation at 94°C for 3 min; four cycles of 94°C for 20 s, 62°C for 30 s, and 72°C for 35 s; eight cycles of 94°C for 20 s, 60°C for 30 s, and 72°C for 35 s; 25 cycles of 94°C for 20 s, 58°C for 30 s, and 72°C for 35 s; and last synthesis at 72°C for 10 min.

The above primers were used to amplify the α- and β-chain sequences. The Rag-1 gene was amplified with primers Rag1F (5'-CCAAGCTGCAGACATTCTAGCACTC-3') and Rag1R (5'-CTGGATCCGGAAAATCCTGGCAATG-3').

Cell staining with antibodies and flow cytometry. Cells (10^6) freshly isolated from lymph nodes were stained with antibodies according to a standard Pharmingen protocol and analyzed in a FACScanto flow cytometer (Becton Dickinson, United States) using the FacsDiva program. Dead cells were excluded from the analysis via staining with propidium iodide (PI) and measuring the scatter parameters. At least 50000 events were recorded to characterize the peripheral T-cell populations. The results were analyzed using the WinMDI 2.8 program.

Antibodies. Flow cytometry (FACScanto, Becton Dickinson, United States) was performed with the following antibodies from Pharmingen (United States): anti-CD3 (Clone 145-2C11), anti-Vα11 (Clone RR8-1), anti-Vβ8.3 (Clone 1B3.3), anti-CD4-PE (Cat. No. 09425A), and anti-CD8-APC (Cat. No. 01049A). To stain dead cells, we used 7.5×10^{-5} M PI (Sigma, United States).

Functional testing of T-cell TCRs of transgenic animals for activation by immobilized antibodies specific for the ε molecule of the CD3 complex. Tests were performed with 5 μg/ml antibodies, which were diluted with sterile Tris-borate. The antibody solution (40 μl) was placed into wells of a 96-well flat-bottom plate, and the plate was incubated at 4°C overnight. The wells were washed three times with 200 μl of cold 1× PBS. A cell suspension was added at 100 μl (0.5×10^6 cells) per well, and the plate was incubated for 3 days. The supernatant was tested for IL-2 with the use of the IL-2-dependent CTLL-2 thymoma cell line.

IL-2 assays with CTLL-2 cells were performed according to the original protocol [4] with minor modification. The supernatant of a cell culture was placed in wells of a 96-well round-bottom plate at 100 μl/well. The wells contained $(5–10) \times 10^3$ CTLL-2 cells, which were preliminarily washed from IL-2 three times with PBS. After 18 h, 15 μl of Alamar Blue (Gibco) were added to each well. Cell viability was inferred after 18–24 h from the reduction of the oxidized dye, using a MULTISCAN instrument. The percent reduction was calculated as $[OD^{exp}_{540} - OD^{exp}_{620} \times (OD^{AB}_{540} - OD^{med}_{540})/(OD^{AB}_{620} - OD^{med}_{620})] \times 100\%$, where OD^{exp}_{540} and OD^{exp}_{620} are the optical densities in test wells, OD^{AB}_{540} and OD^{AB}_{620} are the optical densities of 10% Alamar Blue in 100 μl of the medium, and OD^{med}_{540} and OD^{med}_{620} are the optical densities of 100 μl of the medium at 540 and 620 nm, respectively.

Statistical analysis. The mean and mean error were calculated for each test group. The significance was evaluated by Student's *t*-test.

RESULTS

Cloning of the TCR Genes from Autoreactive Hybridoma 7

A method to produce autoreactive T-cell hybridomas has been developed in our lab. $CD8^+$ cells were preliminarily activated in a mixed culture with splenocytes carrying allogeneic K^{bm3} MCH class I molecules on their surface and were fused with BW5147 thymoma cells expressing the CD4 coreceptor transgene. The resulting hybridomas recognized syngeneic splenocytes carrying cognate A^b MHC class II molecules [8]. Particular interest was attracted by hybridoma 7, since sequencing of its TCR α- and β-chain cDNAs revealed two functional α chains that were associated with the same β chain, belonged to the same Vα11 family, and differed only by point mutations of the variable region and by the J segment. The variable region consisted of the V segment TCRADV11S5 and the J segment JDK1 in the first α chain (7A1) and the V segment TCRAV11S7 and the J segment TA144 (JA) in the second β chain (7A2). The variable region of the β chain (7B) had the V segment BV8S3, D segment DB2, and J segment J2S7 (the TCR α- and β-chain cDNA sequences are available from GenBank (http://

www.ncbi.nlm.nih.gov/sites/entrez) under accession numbers FJ040209, FJ040208, and FJ040207).

Specificity of the Autoreactive TCR of Hybridoma 7

To study the specificity of the autoreactive TCR of hybridoma 7, the full-length cDNAs for the two α chains and the β chain were cloned in retroviral vectors. Viruses carrying the full-length cDNAs for the TCR α- and β chains and the CD4 and CD8 coreceptors were used to infect 4G4 thymoma cells, which did not express their own genes for TCRs and coreceptors, but were capable of expressing the genes for CD3 molecules, essential for the functioning of the TCR/CD3 complex. The TCR and coreceptors were detected using specific antibodies. The TCR functionality was inferred from the IL-2 production by transfected cells upon activation with anti-CD3 ε antibodies sorbed on plastic. Transfectants were activated using splenocytes of C57BL/6 mice, which expressed the genes for the A^b MHC class II molecule; splenocytes of C57BL/6J-$H2^{bm12}$ mice, which differed from C57BL/6 mice in having three point mutations in the α chain of the A^b molecule (amino acid residues 67, 70, and 71); and fibroblasts expressing the gene for the K^{bm3} molecule, which differed from the K^b MHC class I molecule in having two point mutations of residues 77 and 89. The efficiency of activation was inferred from the IL-2 production.

Analysis of the transfectants producing chain 7A1 associated with the β chain showed that the resulting TCR was autoreactive toward cognate MHC class II molecule, while α chain 7A2 conferred specificity to the allogeneic K^{bm3} MHC class I molecule (Fig. 1).

Generation of TCR-Transgenic Animals

Hybridoma 7 was used as a source of an autoreactive TCR for constructing transgenic mice. We used the pTα and pTβ cassette vectors, which were kindly provided by D. Mathis (Harvard University, Boston, United States). The vectors have been designed specially for expressing the rearranged TCR genes in transgenic animals and already have the promoters, enhancers, and regulatory elements essential for targeted expression of a transgenic TCR in T cells. In addition, the vectors have unique restriction sites, which serve to clone the chain genes and to remove prokaryotic DNA before injecting the construct into ovales [3]. The pTα and pTβ cassettes contain the constant region of the TCR α- or β-chain, respectively. Thus, it was necessary to clone the rearranged genomic DNA region from hybridoma 7. The region was amplified with a forward primer directed to the V region and a reverse primer directed to the J segment.

The cloning of the 7A1 α- and β-chain genes from hybridoma 7 is illustrated in Fig. 2. The *Sma*I site was introduced in the α-chain forward primer, which included the start codon. The reverse primer was complementary to an intronic region located approximately 200 bp downstream of the J-segment end (which is necessary for a subsequent splicing of the J segment with the C region) and contained the *Sac*II restriction site. Likewise, the rearranged VDJβ segment of genomic DNA was amplified with a forward primer containing the *Xho*I site and a reverse primer containing the *Sac*II site. The amplification products were initially cloned in the pTZ57RT/A vector. This vector was more convenient for the cloning and sequencing of PCR products than pTα and pTβ, since the products were relatively large and tended to recombine to yield shorter products.

Characterization of Transgenic Animals

We decided to construct transgenic mice that expressed either α or β chains of hybridoma 7 in order to estimate their separate contributions to TCR autoreactivity. The constructs obtained for the α chain were injected in ovales of F_1 (CBA × C57BL/6) mice fertilized with males of the same strain, and the surviving zygotes were grafted to pseudopregnant recipients.

The presence of the transgenic α or β chain was verified by PCR. In the case of the TCR α chain, we obtained five independent transgenic mouse strains: 7A1_3048, 7A1_3000, 7A1_2997, 7A1_2993, and 7A1_3069 (Fig. 3). Of these, only one (7A1_2997) was capable of vertical transmission of the transgene to the progeny. In the case of the β chain, we obtained two independent transgenic strains, 7B_1 and 7B_2, the former being capable of transgene transmission. To estimate the portion of cells producing the transgenic α or β chain in the total T cell population, lymph node cells were stained with monoclonal antibodies against Vα11, Vβ8.3, and CD3. Approximately 80% of 7A1-transgenic (7A1tg) lymph node T cells had the Vα11 chain, and approximately 92.7% of 7B-transgenic (7Btg) lymph node T cells produced Vβ8.3 (Fig. 4). This finding indicated that the transgenes were functional and were expressed in transgenic mice, that the products were successfully folded and associated with the endogenous TCR chains, and that the corresponding T cells are efficiently involved in selection in vivo. The staining intensity of the CD3 complex did not differ between wild-type and transgenic mice, but the proportion of $CD3^+$ cells in lymph nodes of 7A1tg mice was half as high as in wild-type mice (data not shown).

Functional Properties of T Cells of TCR-Transgenic Animals

To study whether T cells of the transgenic mice were functional, lymph node cells were activated with anti-CD3 ε antibodies sorbed on plastic. Figure 5a shows the reduction of Alamar Blue by IL-2-sensitive CTLL-2 cells incubated with various doses of recom-

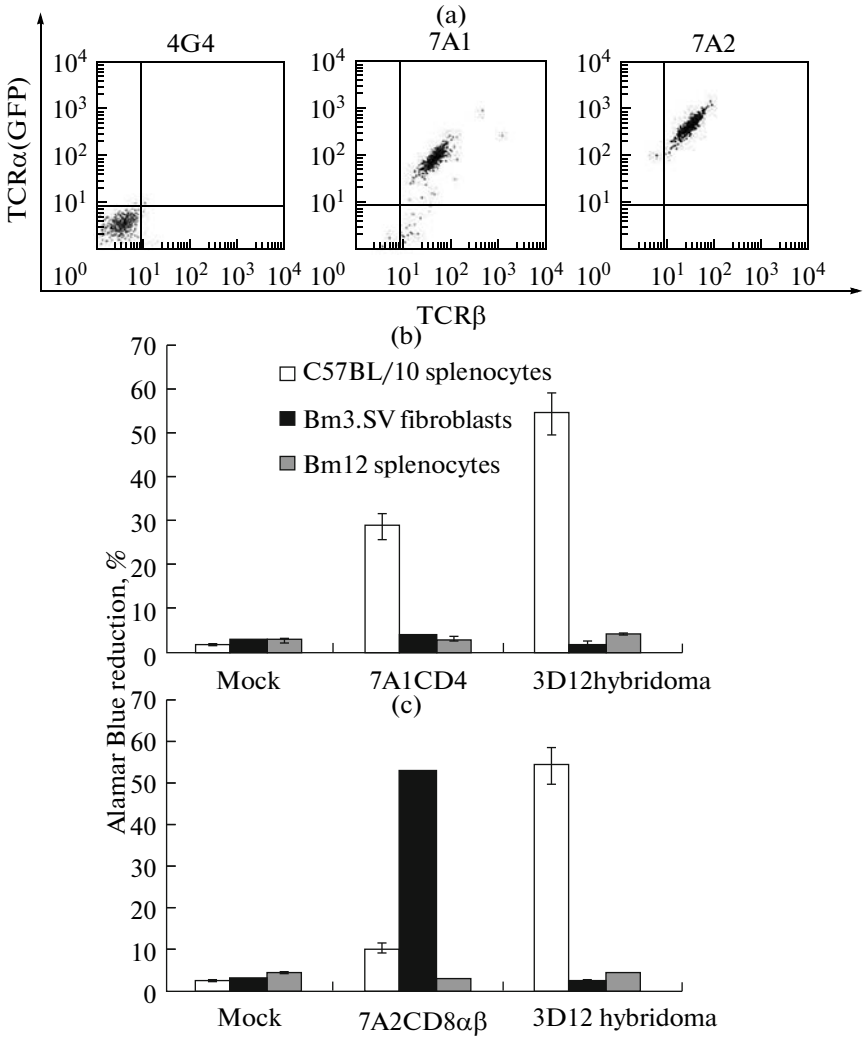

Fig. 1. Presence and specificity of TCRs on the membrane of 4G4 thymoma cells after retroviral transduction of genetic constructs coding for the TCR α and β chains of hybridoma 7. (a) Flow cytometric analysis of intracellular GFP and TCRβ on the surface of 4G4 thymoma cells (control) and 7A1 and 7A2 4G4 transfectants. Abscissa, GFP fluorescence intensity. GFP results from transduction of the TCR α-chain gene within a vector carrying the IRES-GFP gene. Ordinate, intensity of cell staining with antibodies against TCRβ. TCR β is exposed on the membrane of transfected cells as a result of its correct folding and association with TCR α chains. (b, c) IL-2 synthesis in (b) 4G4 thymoma cells transfected with the β chain (TCRBV8S3) in combination with the α chain 7A1 (TCRAV11S5) of hybridoma 7 and the CD4 coreceptor and (c) 4G4 thymoma cells transfected with the β chain (TCRBV8S3) in combination with the α-chain 7A2 (TCRAV11S4) of hybridoma 7 and the CD8 coreceptor. The IL-2 production was assayed after cell stimulation with splenocytes of C57BL/10 mice (open columns), bm3.SV fibroblasts (black columns), and splenocytes of bm12 mice (gray columns). Nontransfected 4G4 cells were used as a negative control. T cells of hybridoma 3D12, which is specific to the A^b MHC class II molecule, were used as a positive control. Abscissa, cell variant. Ordinate, Alamar Blue reduction by CTLL-2 cells, %.

binant mouse IL-2. T cells of our transgenic animals produced IL-2 in response to stimulation if anti-CD3 antibodies (Fig. 5b). The IL-2 production in T cells of 7Btg mice was comparable with that of wild-type mice. The IL-2 production in T cells of 7A1tg mice was lower, which was possibly due to the lower content of $CD3^+$ cells in their lymph nodes. Flow cytometry of lymph node cells from transgenic animals was performed before and after treatment with anti-CD3 antibodies and showed that the portion of $V\alpha11^+$ cells increased from 22.96 to 93.05% after incubation in the case of 7A1tg mice. The portion of $Vb8.3^+$ cells increased from 56.83 to 92.29% in the case of cells isolated from 7Btg mice. These findings indicate that the transgenic TCRs of our animals were functional.

DISCUSSION

One of the most intriguing features of the immune system is its ability to recognize "non-self" while pro-

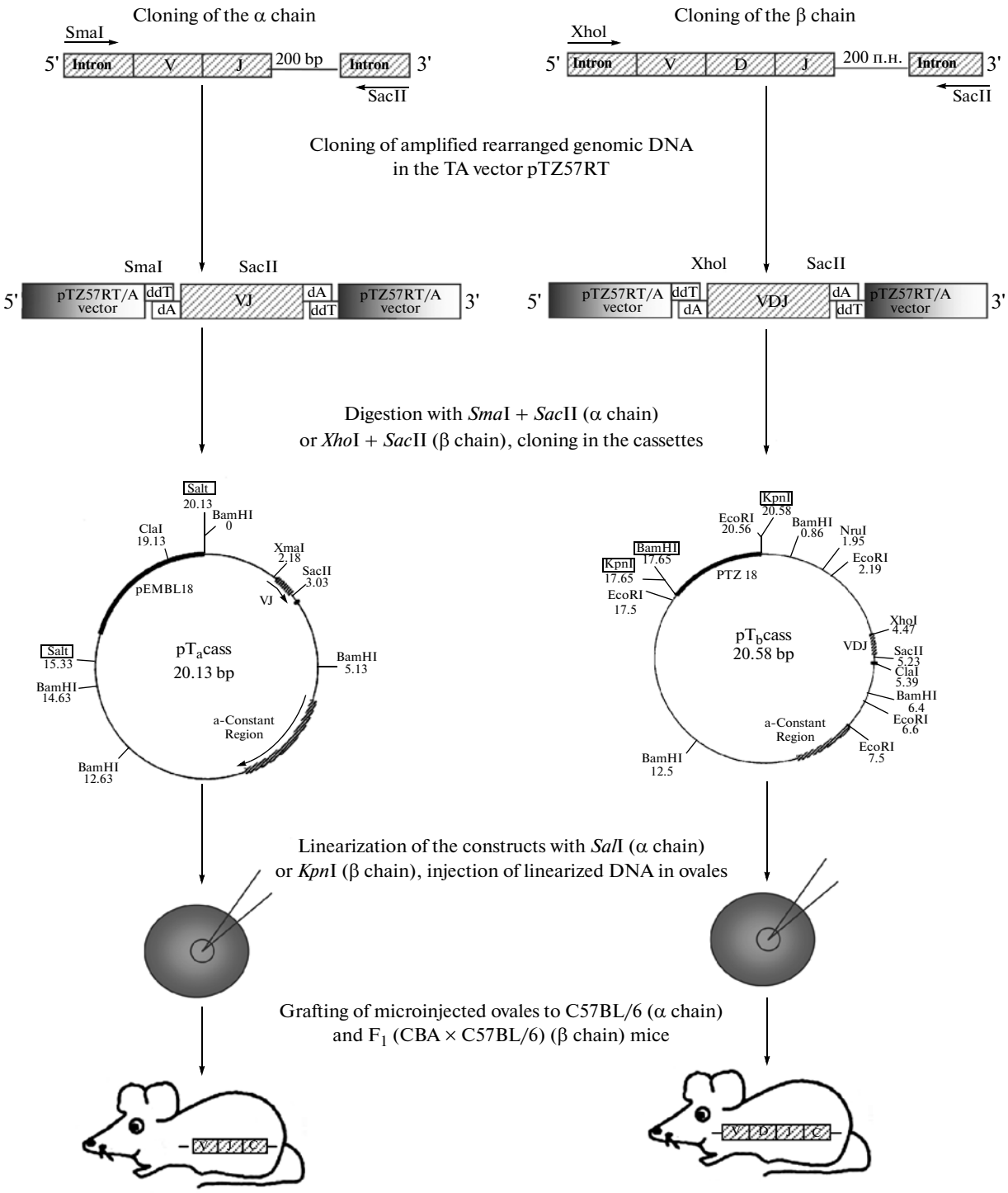

Fig. 2. Cloning of the TCR α- and β-chain sequences in pTα and pTβ and generation of TCR-transgenic animals [3].

ducing no response to "self." This ability is vital because the immune system is continuously exposed to cognate antigens, and a response to them might damage the body. At the same time, when alien antigens find their way into the body, the immune system must be capable of destroying them and their carriers: bacterial toxins and microorganisms or pathogenic viruses and virus-infected cells. Characteristics of T-cell selection are a central problem in understanding the mechanisms of recognizing "self" and "non-

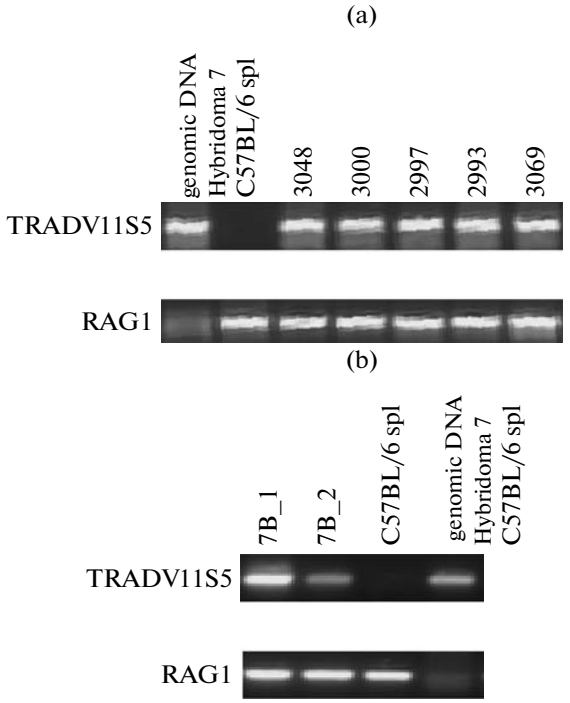

Fig. 3. PCR-based analysis of genomic DNAs from mice transgenic for the α (7A1) or β chain of the autoreactive TCR of hybridoma 7. PCR detected the appearance of (a) the α-chain (TCRAV11S5) sequence in 7A1 mice and (b) the β-chain (TCRBV8S3) sequence in 7B mice. PCR with primers directed to the RAG1 recombinase gene was used as a positive control.

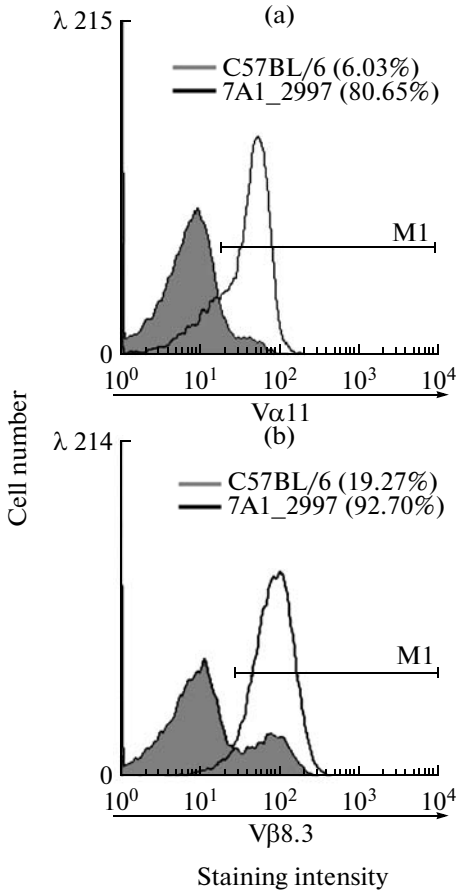

Fig. 4. Flow cytometric analysis of the expression of the transgenes coding for the α and β chain of the autoreactive TCR of hybridoma 7 in 7A1_2997 and 7B_1 transgenic mice. (a) Staining of $CD3^+$ T cells from lymph nodes of mice transgenic for the α chain 7A1 of hybridoma 7 with antibodies specific for the Vα11 family. (b) Staining of $CD3^+$ T cells from lymph nodes of mice transgenic for the β chain of hybridoma 7 with antibodies against the TCRBV8S3 family. The results obtained for $V\beta11^+$ cells of wild-type and transgenic mice are shown with gray filling and a solid line, respectively. Ordinate, staining intensity; abscissa, cell number.

self" by the immune system and the causes of autoimmunity.

A method to produce autoreactive T-cell hybridomas has been developed in our lab with the use of T cells from wild-type mice, since their thymocytes proceed through all differentiation steps without any effect of genetic changes on cell selection within the thymus. This provides an opportunity to isolate and to study the autoreactive receptors that are normally produced in mice. Our model is far more advantageous than genetically modified animals, which usually serve as a source of autoreactive T cells [8]. We constructed transgenic mice producing the α- and β chains of the autoreactive TCR of hybridoma 7. In α chain-transgenic mice, only 80% of peripheral T cells produced the Vα11 β-chain of the autoreactive TCR (7A1). This may be explained by two factors. First, transgenic mice were obtained using wild-type mice with the intact RAG proteins, which are responsible for recombination of the endogenous V(D)J segments of TCRs [12]. Hence, rearrangements of endogenous α chains were possible in T cells of the transgenic mice. Such rearrangements might lead to the presence of two TCRs on the surface of one T cell or might decrease the portion of the transgenic Vα11 chain because endogenous α chains compete with the transgenic chain for the β chain, whose amount in the cell is limited [13, 14].

Second, the transgenic Vα11 β-chain is a component of an autoreactive TCR. The surface density of autoreactive TCRs may decrease during selection [15, 16]. A decrease in the density of the second TCR α chain is known as phenotypic allelic exclusion. This situation is observed not only in wild-type mice, but also in genetically modified mice carrying two transgenic TCRs [17, 18]. It is quite likely that the second TCR, which competes for intracellular signaling molecules, increases the activation threshold of T cells, thus helping autoreactive T cells to evade negative selection. A possible physiological role of T cells expressing two TCRs is possibly related to the circumstance that expression of an additional TCR increases the possibilities of both positive and negative selection [19].

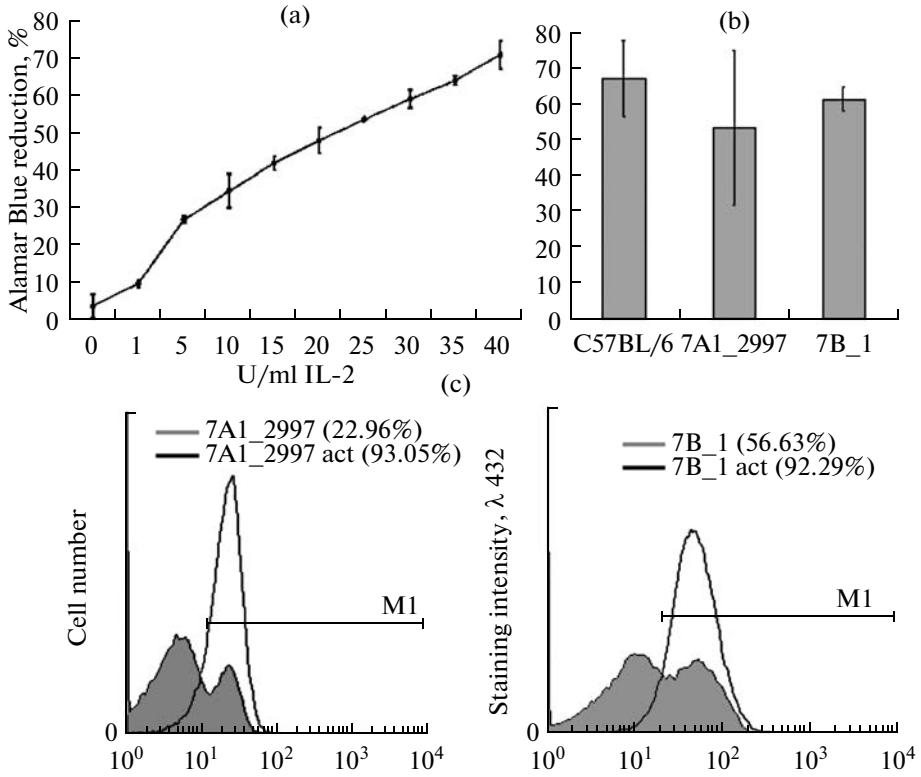

Fig. 5. Functional activity of T cells isolated from transgenic mice and incubated with anti-CD3 antibodies. (a) Alamar Blue reduction by IL-2-dependent CTLL-2 cells incubated with various doses of recombinant mouse IL-2. (b) IL-2 production by T cells from lymph nodes of 7A1_2997 and 7B_1 mice, which are transgenic for the α (7A1) or β chain of the autoreactive TCR of hybridoma 7, respectively, in response to activation with anti-CD3 ε antibodies. T cells of transgenic mice produce IL-2 in response to stimulation with anti-CD3 antibodies. Abscissa, mouse strain and cell lines; ordinate, Alamar Blue reduction by CTLL-2 cells, %. (c) Flow cytometry of cells isolated from lymph nodes of transgenic mice and stained with antibodies to the Vα11 and Vβ8.3 chains before and after incubation with anti-CD3 antibodies. The results obtained for $V\alpha11^+$ and $V\beta8.3^+$ cells before and after incubation with anti-CD3 antibodies are shown with gray filling and a solid line, respectively. Ordinate, cell number; abscissa, staining intensity.

In contrast to mice transgenic for an α chain, rearrangements of the endogenous β-chain locus are usually blocked in mice expressing a transgenic β chain [20–22]. T cells of 7Btg mice were not all positively stained for the Vβ8.3 chain probably because T-cell precursors did not all express the transgene.

Our transgenic mice provide a convenient model for studying various processes underlying the T-cell response, including the formation of T-cell specificity for syngeneic and allogeneic MHC–peptide complexes. This field of research includes several basic problems of the TCR interaction with MHC molecules. One question is whether the recognition of the MHC molecules by TCRs is an innate property or it is a result of thymocyte selection. What are the particular contributions of the CDRs of the α and β chains to the recognition of MHC molecules? How do T cells choose the differentiation lineage during their maturation in the thymus and after activation on the periphery? How does tolerance to cognate antigens form, and why some T cells lack it? How do autoreactive T cells evade negative selection in the thymus? Mice transgenic for autoreactive TCRs can serve as models for studying the development and treatment of various autoimmune disorders similar to those in humans. Moreover, human TCRs are capable of transmitting the signal through mouse CD3, which makes it possible to construct transgenic mice that carry human autoreactive TCRs and MHC molecules [23, 24]. When means are found to regulate the specificity of autoreactive T cells, it will be possible to treat cancer by turning autoimmunity against cancer cells. Such attempts are made in foreign labs via transduction of mouse TCRs specific to tumor antigens into human T cells [25, 26].

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research (project nos. 06-04-48009, 07-04-00567, and 08-04-00563), ZAO Protek Introduction Center, and the Transgenbank Foundation of the Institute of Gene Biology.

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