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Immunology

Exogenous *Mtv-7* superantigen transgene expression in major histocompatibility complex class II I-E$^-$ mice reconstituted with embryonic stem cell-derived hematopoietic stem cells

(minor lymphocyte stimulatory antigen 1)

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ABSTRACT **Direct genetic manipulation of hematopoietic cells is limited by the lack of an established hematopoietic stem cell line. It has been demonstrated that embryonic stem (ES) cell ↔ tetraploid embryos are completely ES cell-derived and that fetal liver (FL) cells from these embryos support hematopoiesis in lethally irradiated recipients. In this report, we demonstrate that FL cells from ES cell ↔ tetraploid embryos support normal lymphopoiesis and T-cell repertoire development. Moreover, the introduction of the *Mtv-7* superantigen transgene coding for minor lymphocyte stimulatory antigen 1 into murine hematopoietic cells via reconstitution with ES cell ↔ tetraploid FL cells demonstrates that this method can effectively confer stable genetic changes into the hematopoietic tissues without going through the germ line. Long-term and secondary reconstitution with ES cell ↔ tetraploid FL cells expressing the *Mtv-7* superantigen transgene clonally deleted minor lymphocyte stimulatory antigen 1-reactive T-cell receptor $V_\beta6^+$, -8.1$^+$, and -9$^+$ T cells, but not $V_\beta7^+$ T cells, in H-2^b (I-E$^-$) mice. This model system will be extremely important for analyzing structure–function relationships of molecules involved in proliferation, differentiation, and selection of hematopoietic cells *in vivo* and for examining hematopoiesis-specific effects of mutations that are lethal during embryogenesis.**

Hematopoietic stem cells (HSCs) differentiate and give rise to all myeloid and lymphoid cells throughout the entire life of the animal (1). Genetic alteration of the HSC thereby has the potential to alter all of the cells in these lineages. Although retroviral vectors have been used to introduce genes into HSCs, there are a number of limitations, including low levels of gene expression and poor infection efficiency (2). Moreover, null mutations cannot be directly generated using HSCs. Thus, the ability to genetically alter HSCs without generating transgenic mice is limited by the lack of an established HSC cell line.

We have demonstrated that embryonic stem (ES) cells aggregated with tetraploid embryos (ES cells ↔ tetraploid embryo aggregates) develop into viable embryos in which the embryos are entirely ES cell-derived and most of the extraembryonic cell lineages are tetraploid-derived (3). Although the offspring die perinatally, the organs of the viable embryos can be used as a source of completely ES cell-derived tissue. The fetal liver (FL) is the primary site of hematopoiesis during the second half of gestation (4, 5). Long-term reconstitution of hematopoiesis in lethally irradiated recipients by ES cell-derived FL cells has been demonstrated (6). However, no detailed analysis of the lymphoid cells was performed in our initial report. Further, the ability of genetically manipulated ES cells to generate these embryos and the resultant phenotypic changes in animals reconstituted with ES cell-derived FL cells has not been demonstrated. In this report we show that a gene transferred to the ES cells can be expressed in hematopoietic cells of the animals reconstituted with the ES cell-derived FL cells. The transgene chosen for this study encodes a minor lymphocyte stimulatory antigen (Mls) that is critically involved in intrathymic selection of developing thymocytes (7, 8) and in conferring resistance to exogenous mouse mammary tumor virus (MMTV) infections in mice (9).

Mls activate a large proportion of T cells via specific V_β chains of the T-cell receptor (TCR) and are classified as superantigens (sag). Recently, it has been demonstrated that Mls are encoded by the open reading frames of the long terminal repeat of MMTV (7, 8). Specifically, Mls-1 is a 45-kDa type II transmembrane glycoprotein encoded by endogenous MMTV provirus *Mtv-7* sag gene (10–13). Mls-1 expression in association with major histocompatibility complex (MHC) class II I-E molecules results in the intrathymic clonal deletion of Mls-1-reactive $V_\beta6^+$, -7$^+$, -8.1$^+$, and -9$^+$ T cells *in vivo* (14–18). The ability of Mls antigens to be presented by I-A remains controversial. Strong Mls stimulators such as Mls-1 can be presented by MHC I-A molecules *in vitro* but much less efficiently than by I-E molecules (8). Studies with backcross inbred, recombinant animals (19) and bone marrow (BM) chimeras (20) indicate that there are varying degrees of partial deletion in $V_\beta6^+$ and $V_\beta8.1^+$ T cells when Mls-1 is presented by H-2^b (I-E$^-$) MHC molecules *in vivo*. *Mtv-7* sag transgene expression in H-2^b mice would directly address this issue and clarify the role of I-A in Mls-mediated tolerance induction.

To this end, ES cell lines constitutively expressing *Mtv-7* sag transgene were used to generate ES cell ↔ tetraploid embryo aggregates. The resultant embryos were 100% ES cell-derived. FL cells from embryonic day (E) 15.5 embryos were then used to reconstitute lethally irradiated adult recipients. E15.5 FL cells from unmanipulated ES cells supported normal lymphopoiesis, specifically the generation of the normal TCR repertoire *in vivo*. Importantly, *Mtv-7* sag gene expression in H-2^b (I-E$^-$) animals modified the TCR repertoire and demon-

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Abbreviations: BM, bone marrow; ES, embryonic stem; FL, fetal liver; HSC, hematopoietic stem cell; Mls, minor lymphocyte stimulatory antigen(s); RAG-2, recombination-activating gene 2; sag, superantigen(s); TCR, T-cell receptor; SN, spleen; LN, lymph node; THY, thymus; E, embryonic day; MMTV, mouse mammary tumor virus; MHC, major histocompatibility complex; GPI, glucose phosphate isomerase.

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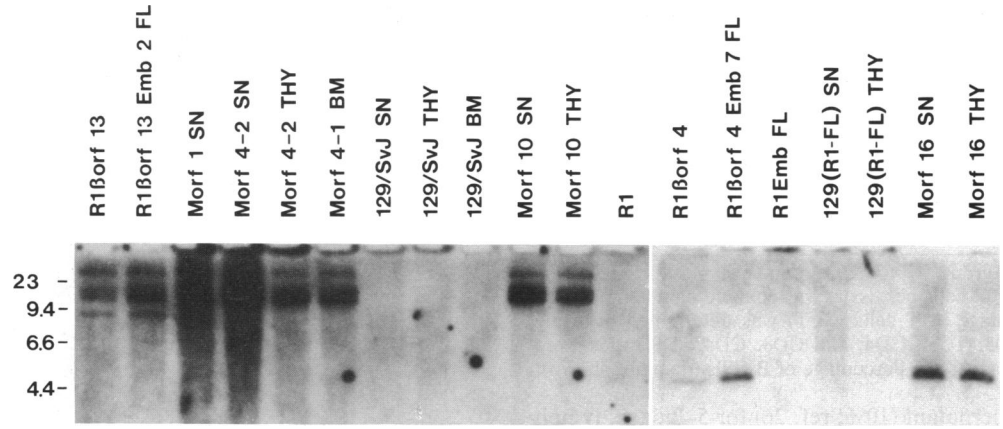

FIG. 1. Detection of the transgene in the hematopoietic cells of ES cell-derived FL-reconstituted mice. Genomic DNA was prepared from the ES cell lines, FL, and lymphoid organs from the reconstituted mice. Southern blot analysis was performed as described (24), and the blots were probed with the *Bal* I–*Bam*HI fragment of the neomycin phosphotransferase cDNA. 129 (R1-FL) were mice reconstituted with FL cells from tetraploid embryos generated with parental cell line R1. The sizes of the DNA bands (in kb) are indicated on the left. Morf 1, 4-1, 4-2, 8, and 10 are group A; Morf 16 is group B; and 129 and R1-FL reconstituted mice are group C. One integrant in R1βorf 13 was never detected in the recipients, suggesting that there was a second clone, which did not contribute to hematopoiesis.

strated that the Mls-specific V_β^+ T cells have varying avidities for Mls-1 associated with I-A molecules.

METHODS AND MATERIALS

Generation of ES Cell Lines. R1 ES cells [129/Sv; H-2^b (I-E^-), Mls-1^-; ref. 21] were maintained and electroporated as described (22) with the pHβA-ORF vector (13). The open reading frame region of the *Mtv-7* sag gene was inserted into the pHβAPr-1-neo expression vector (23), which contains the human β-actin promoter/enhancer and neomycin phosphotransferase cDNA driven by the simian virus 40 early promoter and has been shown to give high expression *in vitro* (13). The cells were selected for neomycin phosphotransferase expression with G418 (200 μg/ml, dry weight; GIBCO). Colonies were picked and expanded, and expression of the *Mtv-7* sag gene was tested by RNA dot blot analysis. Clones showing high expression, R1βorf 13 and 4, were used for the *in vivo* studies.

Mice. 129/SvJ [H-2^b, Mls-1^-, glucose phosphate isomerase (GPI) a/a] mice were purchased from The Jackson Laboratories. CD-1 (Mls-1^-, GPI b/b) mice were bred at our institute. Mice used for FL and BM reconstitutions were 4–8 weeks of age. The animals were maintained in accordance with the Mount Sinai Hospital guidelines.

Generation of Tetraploid Embryos. Tetraploid embryos were generated as described (3). Briefly, 2-cell-stage embryos were flushed from superovulated, fertilized CD-1 females (22). The embryos were oriented and were given a single square electrical pulse of 30 V for 100 μs across 250 μm (CF-150; Biochemical Laboratory Service, Budapest), which resulted in blastomere fusion. The embryos were cultured overnight at 37°C in M16 medium to reach the 4-cell stage. ES cells were gently trypsinized, and 5–10 loosely connected cells were placed between two tetraploid embryos and cultured overnight. The aggregates were transferred into 2.5 day pseudopregnant females with the day of transfer considered E2.5.

FL and BM Reconstitution. 129/SvJ mice were irradiated (10 Gy) and injected i.v. with E15.5 FL (2–3 × 10^6 cells per mouse) or BM (4 × 10^6 cells per mouse). Animals were analyzed 3–9 months postreconstitution.

Southern Blot Analysis. Genomic DNA was isolated from the indicated organs and ES cell lines and analyzed for the presence of the transgene as described (24, 25). After the DNA was transferred, the filters were hybridized with an [α-^{32}P]dCTP (Amersham)-labeled neomycin phosphotransferase probe (*Bal* I/*Bam*HI fragment of the PGKneo vector). Some of the blots were stripped and rehybridized with a β-actin probe to normalize for the amount of DNA as described (24).

RNA Analysis. *Mtv-7* sag mRNA transcripts were detected by PCR using *Mtv-7*-specific oligonucleotide primers: 5′-GAAGGAAAAAGGAGTGTGT and 3′-ATCGAAGC-CAACGCGACCCCC (J.K., B.H., and N.H., unpublished results). RNA (2 μg) was reverse transcribed with pd(N)$_6$ (Pharmacia) and avian myeloblastosis virus (Boehringer Mannheim). The resulting cDNA was amplified with the primers and *Taq* polymerase (Cetus) using the following program: 1 min at 94°C, 1 min at 57°C, 1.5 min at 72°C for 35 cycles. One-fiftieth of the products was analyzed by Southern blot analysis and hybridized with the *Mtv-7* sag probe (13).

Flow Cytometry. Cell suspensions from spleen (SN), splenocytes stimulated with Con A (4 μg/ml; Pharmacia) and

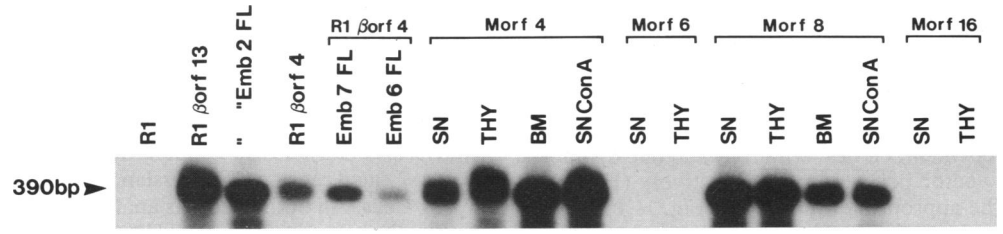

FIG. 2. Detection of exogenous *Mtv-7* sag gene expression. Transgenic RNA was detected by PCR, using oligonucleotides specific for the *Mtv-7* sag gene. Total RNA (2 μg) was reverse transcribed and amplified as described above. One-fiftieth of the product was analyzed by Southern blot analysis, and the blot was hybridized with the *Mtv-7* sag probe (13). Faint bands can be seen in animals reconstituted with FL cells from embryos generated with the R1βorf 4 ES cell line upon long exposure.

Table 1. Normal T-cell development in ES cell-derived FL-reconstituted animals

Group*	Donor cells	SN† % T	SN† No. × 10^{-7}	LN % T	LN No. × 10^{-6}	THY % T	THY No. × 10^{-7}
A	R1βorf 13	24	2.3	56	7.3	98	6.9
B	R1βorf 4	20	2.0	54	8.6	99	6.5
C	129	27	2.4	64	8.0	98	6.5

*Group A, n = 9; group B, n = 10; group C, n = 10.
†The total number of cells per tissue was counted on a hemocytometer. The percentage of T cells (% T) was determined by using antibodies to CD3, TCR, CD4, and CD8. CD4/CD8 ratios were normal (2–3:1), as was the percentage of B cells (data not shown).

interleukin 2 supernatant (10%; ref. 26) for 5–7 days, lymph node (LN), and thymus (THY) were prepared. Antibodies specific for the following cell surface antigens were used: CD4-phycoerythrin (PE), CD8-fluorescein (FITC), and Thy1.2-biotin (all from Becton Dickinson), CD3-FITC, V_β6-biotin, V_β8.1/8.2-biotin, V_β7-biotin, V_β9-biotin, V_β14-FITC, and TCR$\alpha\beta$-FITC (all from PharMingen), and V_β8.2-biotin (F23.2). Biotinylated antibodies were incubated with streptavidin-FITC or streptavidin-PE (Becton Dickinson). The samples were analyzed by using an EPICS V (Coulter Electronics).

Antibody-Mediated T-Cell Proliferation. LN cells were incubated with the following immobilized antibodies: anti-V_β6, anti-V_β7, anti-V_β9, and anti-TCR (0.3 μg per well) and anti-V_β8.1/8.2 (1 μg per well) (J.K. and N.H., unpublished results). Antibodies were incubated in 96-well plates (Nunc) for 2 hr at 37°C, washed with phosphate-buffered saline, and blocked with 10% (vol/vol) fetal calf serum in RPMI for 15 min at 37°C. The cells were cultured (2×10^5 cells per well) for 3 days, and 1 μCi (1 Ci = 37 GBq) of tritiated thymidine was added per well for the last 12 hr of incubation.

RESULTS

Introduction of *Mtv-7* sag Transgene into Mice via ES Cell ↔ Tetraploid Embryo FL Cell Reconstitution. The tetraploid embryos were generated with ES cell line R1 and two R1 *Mtv-7* sag gene transfectants, R1βorf 13 and R1βorf 4. Viable ES cell ↔ tetraploid E15.5 chimeras were obtained from 25–30% of the ES cell ↔ tetraploid aggregates transferred. The embryos were completely derived from ES cells, as determined by GPI analysis (data not shown; refs. 3 and 6). Animals were analyzed 3–9 months postreconstitution to examine long-term expression of the transgene in hematopoietic cells. SN, THY, LN, and BM cells were analyzed. Animals reconstituted with R1βorf 13 FL cells were designated Morf 1, 4, and 8–12. Morf 4-1 and 4-2 are secondary BM chimeras using Morf 4 BM cells (5 months postreconstitution) as donor cells (group A). R1βorf 4 FL cells were used to reconstitute Morf 2, 3, 5, 6, and 14–18 mice (group B). Age-matched control 129/SvJ and 129/SvJ reconstituted with R1-derived FL cells were also analyzed. There was no difference in the lymphocyte populations between these two sets of control mice, and the data was combined into group C (n = 10).

Southern Blot Analysis. Genomic DNA from the FL and the hematopoietic tissues of the reconstituted animals was analyzed for the presence of the transgene. ES cell lines R1βorf 13 and 4 were independent clones, with four and one distinct integrants of the vector per genome, respectively (Fig. 1). The presence of the appropriate transgenes (Fig. 1) and GPI isoforms (data not shown) in all of the hematopoietic tissues of the recipient animals demonstrated that the animals were reconstituted with ES-derived FL hematopoietic stem cells. One of the integrant bands in the R1βorf 13 and embryo 2 FL was not present in the FL-reconstituted animals, possibly due to a second clone in R1βorf 13 cell line that did not contribute to hematopoiesis in the recipient animals.

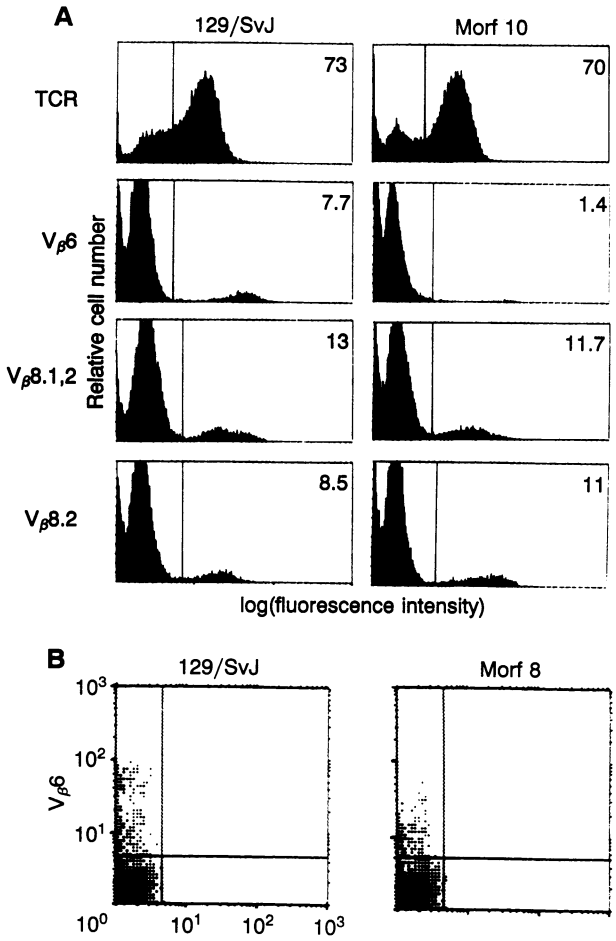

FIG. 3. Proportion of LN T cells expressing V_β6, V_β8.1, and V_β8.2 in ES cell-derived FL-reconstituted mice. Cells from the mesenteric LN (*A*) and THY (*B*) were stained with antibodies specific for the cell surface antigens indicated. Ten thousand cells were counted per sample. In *B*, the percent $V_\beta6^+$ thymocytes (TCR^{hi} and TCR^{low}) was 6% and 3% in 129/SvJ and Morf 8 mice, respectively.

***Mtv-7* sag Gene Expression.** ES cell, R1βorf 13, and FL cells from all of the embryos generated with R1βorf 13 (n = 6) expressed the *Mtv-7* sag transgene (Fig. 2). All of the mice reconstituted with R1βorf 13 FL cells (n = 9) were positive for the resultant 390-bp band in samples from the SN, THY, splenic T cells (SN ConA), and BM (Morf 4 and 8; Fig. 2). Also, *Mtv-7* sag mRNA expression was maintained in the secondary recipients Morf 4-1 and 4-2, which received BM cells from Morf 4. No product was detected when samples were treated with RNase A prior to the PCR reaction (data not shown) or in the 129/SvJ or R1-derived FL-reconstituted animals (Fig. 2). Although the R1βorf 4 ES cell line expressed the *Mtv-7* sag gene *in vitro*, gene transcription was decreased in the ES-cell-derived FL cells (n = 9) and was virtually undetectable in the hematopoietic tissues of the FL-reconstituted animals (n = 10) (Morf 6 and 16; Fig. 2). These results suggest that the insertion site of the vector was transcriptionally downregulated during embryogenesis.

***Mtv-7* sag Transgene Expression Deletes Some Mls-Specific V_β^+ T Cells.** All of the animals analyzed had normal numbers of lymphocytes in the SN, THY, and LN and had normal proportions of T (Table 1) and B cells. The expression of the exogenous *Mtv-7* sag gene in 129 mice allowed for the examination of the effects of Mls-1 antigen expression on the formation of the TCR repertoire in the absence of MHC class

Table 2. Summary of fluorescence-activated cell sorting analysis of FL-reconstituted mice

Group*	% of total LN T cells					*Mtv-7* mRNA‡
	$V_\beta 6$	$V_\beta 7$	$V_\beta 8.1$†	$V_\beta 8.2$	$V_\beta 9$	
A	1.5 ± 0.5	3.7 ± 0.7	1.1 ± 0.9	13.8 ± 1.9	0.6 ± 0.2	+
B	10.8 ± 1.4	4.1 ± 1.3	6.8 ± 1.3	11.5 ± 2.1	2.4 ± 0.7	−
C	9.2 ± 0.9	3.3 ± 0.6	6.6 ± 0.6	9.8 ± 1.8	2.3 ± 0.1	−

*Group A, $n = 9$; group B, $n = 10$; group C, $n = 10$.
†The percentage of $V_\beta 8.1^+$ T cells was determined by subtracting the percentage of $V_\beta 8.2^+$ cells from the percentage of $V_\beta 8.1/8.2^+$ cells.
‡Determined by PCR.

II I-E molecules. Representative results from fresh LN and THY cells are given in Fig. 3, and Table 2 is a summary of all of the results.

Expression of the *Mtv-7* sag gene in the hematopoietic cells of R1βorf13 FL-reconstituted and secondary BM-reconstituted mice resulted in a virtually complete deletion of $V_\beta 6^+$, -8.1^+, and -9^+ peripheral T lymphocytes (Fig. 3*A*). There was a small compensatory increase in the $V_\beta 8.2^+$ T cells (Table 2). Interestingly, there was no deletion of $V_\beta 7^+$ T cells in animals expressing the *Mtv-7* sag transgene compared to the control animals (Table 2), the V_β that has been shown to have the lowest avidity for vSAG-7 in association with I-E molecules (18). Identical results were obtained when expanded splenic T cells were analyzed (data not shown).

In the thymus, 2–3% of the TCR^{hi} thymocytes were $V_\beta 6^+$ in the animals not expressing the *Mtv-7* sag gene (groups B and C), whereas the vSAG-7^+ (group A) animals had <0.5% TCR^{hi} $V_\beta 6^+$ thymocytes (Fig. 3*B*). There was no difference in V_β expression in the immature TCR^{low} subset between these groups of mice. Thus, intrathymic negative selection mediated by the BM-derived vSAG-7^+ thymic cells occurred at the transition from TCR^{low} to TCR^{hi} expression.

No phenotypic changes were observed in mice reconstituted with FL cells from embryos generated with R1βorf4 ES cells, which did not express the *Mtv-7* sag gene. The proportions of the V_β^+ T cells in these animals were comparable to those in the control mice (Table 2).

These results indicate that the clonal deletion process does not require MHC I-E expression for three of the four Mls-reactive V_β TCRs. However, $V_\beta 7^+$ T cells were not significantly affected by the *Mtv-7* sag transgene expression in $I\text{-}E^-$ mice. Similar results were obtained when C57BL/6 ($I\text{-}E^-$) blastocysts were used to generate the tetraploids, indicating that the observed deletion of Mls-1-responsive T cells was not due to undetectable numbers of contaminating $I\text{-}E^+$ cells from the CD-1 tetraploids.

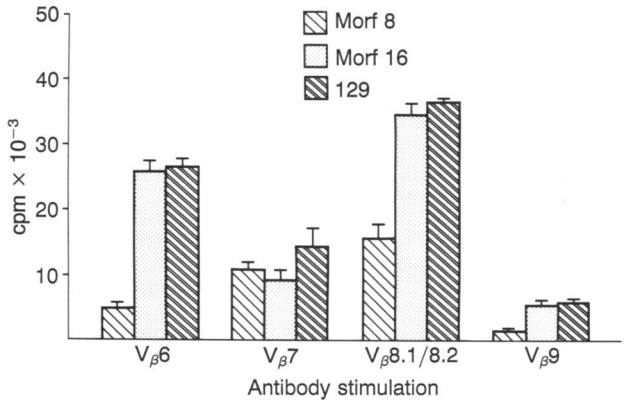

FIG. 4. Antibody proliferation. Representative proliferation data for Morf 8 (group A), Morf 16 (group B), and 129 (group C) are shown. Freshly isolated LN cells were incubated with immobilized antibodies for 3 days and were pulsed with [^{3}H]thymidine for the last 12 hr of culture. Stimulation with Con A and anti-TCR monoclonal antibody gave consistent proliferative responses between and within the three groups of animals.

Antibody Proliferation of Specific V_β-Expressing T Cells. Although $V_\beta 7^+$ T cells were not deleted in the transgenic vSAG-7^+ mice, these cells may have been clonally inactivated by exposure to Mls-1 antigen. To test this possibility, LN cells were stimulated with immobilized anti-TCR antibodies. Representative data are given in Fig. 4. Proliferation in response to anti-$V_\beta 6$, $-V_\beta 8.1/8.2$, and $-V_\beta 9$ monoclonal antibodies in the animals reconstituted with R1βorf 13-derived FL cells (group A) was reduced compared to groups B and C. However, there was no difference in the response to the $V_\beta 7$ monoclonal antibody (Fig. 4), indicating that the $V_\beta 7^+$ T cells were not anergized when vSAG-7 was presented by $H\text{-}2A^b$ *in vivo*.

DISCUSSION

In this report we demonstrate that completely ES cell-derived FL cells support normal lymphopoiesis—specifically, generation of the normal TCR repertoire in inbred mice irradiated and reconstituted with FL cells. Further, genetic manipulation of genes involved in hematopoiesis can be assessed *in vivo* without having to generate transgenic mice. Introduction and expression of the *Mtv-7* sag gene into the hematopoietic tissues of reconstituted animals was efficient and resulted in phenotypic changes in the TCR repertoire. By using this model, gene transfer into the hematopoietic tissues of recipients can be easily accomplished using standard gene transfection technology into ES cells. Gene expression of the transgene in the ES cell ↔ tetraploid embryos and the FL-reconstituted animals, however, will depend upon the site of integration in the genome and possible transcriptional down-regulation during embryogenesis. Our results suggest that high gene expression from a constitutive promoter in E15.5 FL indicates the transgene will be expressed in recipients. Thus FL cells from embryos generated with a number of independent cell lines can be screened quickly for exogenous gene expression prior to animal reconstitution.

Genetic manipulation of the hematopoietic system by the introduction of the *Mtv-7* sag gene into HSCs via ES-derived FL cells is, to our knowledge, the first report of *Mtv-7* sag transgene expression *in vivo*. Several important observations concerning the efficacy of clonal deletion by the constitutively expressed *Mtv-7* sag gene product molecules can be made. First, the avidity of different Mls-specific V_β chains for Mls-1/$I\text{-}A^b$ can be clearly distinguished. We have demonstrated by direct transgene expression of Mls-1 in hematopoietic cells that there is no significant difference in clonal deletion of $V_\beta 6^+$, -8.1^+, and -9^+ T cells in $H\text{-}2^b$ ($I\text{-}E^-$) mice compared with $I\text{-}E^+$ animals (14–17). Previously, Webb and Sprent (27) have shown that $V_\beta 6^+$ T cells are deleted in D1.LP mice ($H\text{-}2^b$, $I\text{-}E^-$, Mls-1^+) to levels similar to the *Mtv-7* sag transgene-expressing mice described in this report. Unlike $I\text{-}E^+$ mice, clonal deletion of $V_\beta 6^+$ T cells in D1.LP mice is critically dependent on B cells and occurs significantly slower than in $I\text{-}E^+$ mice (27). These and other results suggest that V_β-Mls-1/I-A recognition is a low-avidity interaction (19, 28),

and there may be a gene dose effect similar to that observed in exogenous MMTV sag transgenic mice (9). Proportionally, the extent of deletion of $V_\beta6^+$ and $V_\beta8.1^+$ T cells is greater than that seen with $V_\beta9^+$ T cells. Further, $V_\beta7^+$ T cells are neither clonally deleted nor clonally inactivated by the vSAG-7/I-A^b ligand. Thus, the *in vivo* avidity of Mls-1-specific V_β chains for vSAG-7/I-A^b can be ranked as $V_\beta6$, $V_\beta8.1 > V_\beta9 >>> V_\beta7$.

Models of Mls antigen interaction with MHC class II and TCR have been proposed (29). The method described in this paper can be used to elucidate the molecular interactions between MHC class II, Mls, and TCR molecules *in vivo*. Two regions unique to the *Mtv-7* sag gene product have been identified and are proposed to be important for MHC class II binding and specific V_β TCR recognition (13). Specific amino acid substitutions within these regions could be tested for the ability to interact with MHC class II molecules and to stimulate Mls-1-reactive T cells *in vivo* using this technique. Similarly, the effect of mutations in V_β chains and the role of V_α chains (30, 31) in Mls reactivity in mice of various genetic backgrounds can be assessed. ES cell ↔ tetraploid embryos constitutively expressing the *Mtv-7* sag gene during ontogeny can also be used to examine the hypothesis that neonatal Mls-reactive T cells are only deleted day 10 after birth due to the lack of expression of the vSAG antigen (32, 33).

Recently, another method to study lymphopoiesis *in vivo* called recombination-activating gene (RAG) 2-deficient blastocyst complementation was reported (34). ES cells are injected into RAG-2-deficient blastocysts, generating somatic chimeras in which the mature lymphocytes are ES cell-derived. Although useful, particularly for screening genes postulated to be involved in TCR and immunoglobulin gene rearrangement, this method has several important limitations. First, the animals are somatic mosaics. Thus, lymphocyte development and homing will occur in a heterogeneous microenvironment. This could greatly influence the resultant TCR and immunoglobulin repertoires. Further, experiments are limited to the genotype of the RAG-$2^{-/-}$ mice. Second, the method is restricted to analysis of mature lymphocytes, since myeloid cells, HSCs, and early T and B cell development arise from both ES cells and RAG-2^- blastocysts (34, 35). Third, variability in the B- versus T-cell subpopulations occurs due to variability of the ES cell contribution (34).

The model described in this report has a number of important advantages. The ES cell ↔ tetraploid FL cells can be used to examine genes involved in growth and differentiation of all hematopoietic cell lineages. Embryos are completely ES cell-derived; thus, molecules expressed in the microenvironment necessary for normal hematopoiesis can be examined. Further, the effects of genetic manipulations on fetal development and analysis of gene expression can be assessed directly in the embryos and perinates. In addition, organs from these embryos, such as the fetal THY (36), can be used for fetal organ culture and as a source of other types of stem and precursor cells. The ES cell ↔ tetraploid embryos can be generated with any ES cell line. This will be particularly important as new ES cell lines are generated from numerous inbred wild-type and mutant genotypes. Moreover, this system is ideal for studying mutations that are lethal in late embryogenesis and/or hematopoiesis-specific effects. A vast number of molecules postulated to be involved in the generation of immune responses have been identified. Using ES cell ↔ tetraploid FL reconstitution, multiple genes or a series of mutations of a particular gene can be introduced into hematopoietic system without going through the germ line, thus enabling rapid and efficient analysis of the role and structural requirements of these molecules *in vivo*.

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