T-cell receptor $\gamma$-$\delta$ and $\gamma$ transgenic mice suggest a role of a $\gamma$ gene silencer in the generation of $\alpha\beta$ T cells

(T-cell lineages/allelic exclusion)

ISAO ISHIDA*, SIEF VERBEEK†, MARC BONNEVILLE*, SHIGEYOSHI ITOHARA*, ANTON BERNT†, AND SUSUMU TONEGAWA*

*Howard Hughes Medical Institute at Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139; †The Netherlands Cancer Institute, Division of Molecular Genetics and the Department of Biochemistry of the University of Amsterdam, Amsterdam, The Netherlands

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ABSTRACT A T lymphocyte expresses on its surface one of two types of antigen receptor, T-cell receptor $\alpha\beta$ or T-cell receptor $\gamma$-$\delta$. It has been suggested that $\alpha\beta$ T cells are generated only from precursor T cells that failed to rearrange $\gamma$ and $\delta$ genes in a functional form. However, we found that transgenic mice constructed with functionally rearranged $\gamma$ and $\delta$ genes produce a normal number of $\alpha\beta$ T cells. The transgene $\gamma$ present in these $\alpha\beta$ T cells is repressed apparently through an associated cis DNA element (silencer). We propose that some T-cell precursors are committed to generate $\alpha\beta$ T cells independent of the rearrangement status of their $\gamma$ gene and that this commitment involves activation of a factor(s) that interacts with the $\gamma$ gene-associated silencer.

The search for the genes encoding the antigen receptor of T cells, T-cell receptor (TCR) $\alpha\beta$, led to the identification of a third set of TCR genes, $\gamma$ (1-3), which in time led to the discovery of a second TCR, TCR $\gamma$-$\delta$ (4-6). One interesting issue raised by the discovery of $\gamma$-$\delta$ T cells is their developmental relationship with $\alpha\beta$ T cells. During thymic ontogeny, rearrangement and cell-surface expression of $\gamma$ and $\delta$ genes precede those of $\alpha$ and $\beta$ genes (7-9). In peripheral $\alpha\beta$ T cells, some $\gamma$ genes are rearranged, often but not always, in a nonproductive (i.e., out-of-frame) form (2, 10-12). In contrast, in $\gamma$-$\delta$ thymocytes or peripheral $\gamma$-$\delta$ T cells, $\beta$ genes are almost always in an incompletely rearranged D-J form (D. diversity; J. joining), while $\alpha$ genes are never rearranged (13-15). In addition, cells bearing both TCR $\alpha\beta$ and TCR $\gamma$-$\delta$ or "hybrid" TCRs such as $\beta\delta$ or $\alpha\delta$ heterodimers do not exist or are very rare (8, 9, 16). On the basis of these observations, Pardoll et al. (8) and Allison and Lanier (17) proposed that if the $\gamma$ and $\delta$ gene rearrangements are both productive, the cells proceed to surface expression of TCR $\gamma$-$\delta$, which, in analogy with the immunoglobulin system (18), inhibits further rearrangement of any other TCR gene. $\alpha\beta$ T cells are generated only from those cells that failed to productively rearrange both $\gamma$ and $\delta$ genes.

We tested this model by analyzing transgenic (Tg) mice constructed with a product of rearranged $\gamma$ and $\delta$ genes cloned from a $\gamma$-$\delta$ T-cell hybridoma. Since all T-cell precursors in these mice should have productively rearranged Tg $\gamma$ and $\delta$ genes, the model would predict that generation of $\alpha\beta$ T cells would be blocked. However, we found that a normal number of $\alpha\beta$ T cells is produced in these Tg mice. The $\gamma$ and $\delta$ Tg are transcriptionally silent in $\alpha\beta$ T cells and the repression of $\gamma$ gene expression seems to be mediated by a cis-acting DNA element (a silencer) present in the flanking regions of the $\gamma$ gene. We suggest that some precursor T cells are programmed to repress the expression of $\gamma$ (and also perhaps $\delta$) genes, which may be either productively or nonproductively rearranged, by a mechanism mediated by the silencer element(s) and that it is from these precursor T cells that $\alpha\beta$ T cells are generated.

MATERIALS AND METHODS

Monoclonal Antibodies (mAbs). Anti-TCR $\gamma$-$\delta$ (G10) and anti-clonotypic Kv6.7 (SC10) mAbs have been described (9). Anti-TCR $\alpha\beta$ (I57-597) was a gift from R. Kudo (National Jewish Center for Immunology and Respiratory Medicine, Denver) (19).

DNA Probes and Primers. The V$_{\gamma4}$, I$_{\gamma4}$, and V$_{\delta}$ (V, variable) probes have been described (15). The C$_{\gamma}$ and C$_{\delta}$ (C, constant) probes have also been described (1). The Thy-1 and actin probes were prepared from clone pMT8 (20) and an actin cDNA clone (21), respectively.

Construction of Tg Mice. For the production of $\gamma$-$\delta$ double Tg mice, the inserts of the pB-25-15 and p54+2S cosmid cloned in the pWE15 vector (22) were dissected by Sma I and Not I, respectively, and co injected (4 Âµg/ml each) into fertilized mouse eggs from superovulated (CBA/Bra × C57BL/Li)F$_1$ females that had been mated with F$_1$ males (23). The Tg mice were identified by the tail DNA-blotting method using 32P-labeled V$_{\gamma4}$, I$_{\gamma4}$, and V$_{\delta}$ probes. The $\gamma$ single Tg mice were similarly prepared by using the EcoRI/Sal I insert of the phage λ clone pJ-Λ.

Southern and Northern blot Analyses and Quantitation of Band Intensity. DNA and RNA were purified by the standard procedures and subjected to analysis according to Chomczynski and Sacchi (24) using probes labeled by a random priming method (25). The autoradiographic bands were detected and intensities were quantitated by using a Fuji Bioimage analyzer BA100 (26). This machine detects radioactivity recorded on xerochromatic phosphor upon stimulation by a laser beam and is at least 100 times more sensitive than conventional autoradiography with x-ray film for the detection of 3P. The machine also detects a wide range of radioactivity (50-10^6 dpm) in a linear fashion, enabling highly accurate quantitation of band intensity.

Polymerase Chain Reaction (PCR). Ten micrograms of total RNA from each T-cell hybridoma was incubated with 0.5 ÂµM synthetic 3' primer (GTCTTATAATGTTGAAATCAGA), 0.5 mM dNTPs, 50 ÂµM Tris-HCl (pH 8.3), 10 ÂµM MgCl$_2$, 8 mM dithiothreitol, 2 units of RNasin (Amersham), and 10 units of reverse transcriptase (Amersham) in total vol of 20 ÂµL. After 45 min at 42°C, the reaction mixture was diluted to 200 ÂµL with 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 2.5 mM MgCl$_2$, together with 1 mM synthetic 5' primer (CTCTCTAATCTGA-

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CATCACCTC), 250 μM dNTPs, and 0.5 unit of Thermus aquaticus DNA polymerase (Taq polymerase) (PerkinElmer/Cetus), PCR cycles were run according to ref. 27 with 1 min at 92°C, 2 min at 50°C, and 3 min at 72°C.

Flow Cytometry and Fluorescence-Activated Cell Sorting. Flow cytometry was carried out as described (9) using a FACScan (Becton Dickinson). For the purification of αβ T cells, splenic T cells from γδ-1355 mice were enriched by a nylon wool column and stained with biotin-coupled anti-αβ mAb and phycoerythrin-streptavidin and sorted. The purification of γδ T cells from γδ-1355 mice was carried out as described (28).

Generation of T-Cell Hybridomas. For the generation of αβ and γδ hybridomas, total thymocytes or splenic T cells and CD4−CD8− (double negative) thymocytes were fused with an αβ TCR-negative thymoma BW5147 (29) as described (14).

RESULTS

Construction of γδ Double Tg Mice. Three γδ double Tg mice (γδ-1355, γδ-1314, and γδ-1313) were obtained by coinjection of two cosmID clones, Py4 and Py8-23/15 (Fig. 1), which carry the γ and δ genes, respectively, encoding the TCR of a γδ T-cell hybridoma KN6 (14). The three Tg lines carry a single to a few copies of the injected γ and δ genes (Table 1). Their splenocytes and thymocytes (Table 1) but not livers (data not shown) contain elevated levels of γ and δ RNA of appropriate sizes, suggesting that the Tg are expressed in the lymphoid organs of these mice. The presence of Tg-encoded RNA in the thymus was confirmed by a separate experiment in which synthetic oligonucleotide probes specific to the V-J or V-D-J junctional functional sequences of the Tg were used (data not shown).

An Increased but Still Minor Proportion of Thymocytes and Splenic T Cells Expresses the Tg on the Cell Surface. The total number of thymocytes and splenocytes is equivalent between the Tg mice and their non-Tg littermates (Table 2). Using appropriate mAbs in flow cytometry, we quantitated the γδ TCR and αβ TCR-expressing cells among the various cell populations. Less than 0.2% of thymocytes and <1% of splenocytes from normal mice bear γδ TCRs, and cells bearing KN6 γδ TCRs are too low to be detected in either cell population (Table 2; ref. 9). In contrast, in the γδ Tg mice, 2–3% of thymocytes and 3–4% of splenocytes bear γδ TCRs, of which a large proportion (80–100%) is KN6 clonotype positive (Table 2). These results strongly suggest that the Tg-encoded TCRs dominate the γδ T-cell population. However, γδ T cells still compose a relatively small proportion (<4%) of thymocytes and splenocytes in the Tg mice.

Indeed, analysis with the αβ TCR mAb indicated that neither the absolute number nor the proportion (72–75% of total thymocytes and 22–28% of total splenocytes) of αβ T cells is significantly altered in the thymus and spleen of the Tg mice (Table 2). These thymocytes and splenocytes exhibit a normal expression pattern of CD4 and CD8 glycoproteins.

Table 1. Copy number of Tg and relative level of their transcripts in the various Tg mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Copy number of Tg</th>
<th>Vγ4 transcripts in Thymocytes</th>
<th>Vδ5 transcripts in Thymocytes</th>
<th>Vγ4 transcripts in Splenocytes</th>
<th>Vδ5 transcripts in Splenocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>LM control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γδ-1355</td>
<td>2</td>
<td>5</td>
<td>0.008</td>
<td>0.004</td>
<td>0.032</td>
</tr>
<tr>
<td>γδ-1314</td>
<td>2</td>
<td>1</td>
<td>0.160</td>
<td>0.028</td>
<td>0.352</td>
</tr>
<tr>
<td>γδ-1313</td>
<td>1</td>
<td>5</td>
<td>0.045</td>
<td>0.008</td>
<td>0.025</td>
</tr>
<tr>
<td>γδ-2017</td>
<td>10</td>
<td>10</td>
<td>0.078</td>
<td>0.019</td>
<td>0.454</td>
</tr>
<tr>
<td>γδ-2018</td>
<td>1</td>
<td>6</td>
<td>0.791</td>
<td>0.292</td>
<td>0.433</td>
</tr>
</tbody>
</table>

The copy numbers of the Tg were estimated from the intensity of the Southern blot bands obtained by hybridizing EcoRI-digested liver DNA with the Vγ4 or Vδ5 probe. A known amount of EcoRI-digested py4 and p8-23/15 cosmID DNA electrophoresed side by side with the liver DNA provided the standard. The γ and δ RNAs were detected by the Vγ4 and Vδ5 probes, respectively, and their relative content was estimated from the band intensity using the actin RNA as the internal control. Results are expressed in arbitrary units.
Table 2. Surface expression of γδ TCR and αβ TCR on thymocytes and splenocytes of γδ Tg mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Age, weeks</th>
<th>Total thymocytes</th>
<th>Thymocytes (×10^6) stained with</th>
<th>Total splenocytes</th>
<th>Splenocytes (×10^6) stained with</th>
</tr>
</thead>
<tbody>
<tr>
<td>γδ-1313</td>
<td>9</td>
<td>79</td>
<td>1.26 (1.6)</td>
<td>56.8 (71.9)</td>
<td>6.32 (4.1)</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>78</td>
<td>0.16 (0.2)</td>
<td>55.9 (71.7)</td>
<td>0.00 (0.0)</td>
</tr>
<tr>
<td>LM 8</td>
<td>100</td>
<td>2.66 (2.7)</td>
<td>72.7 (72.7)</td>
<td>4.92 (3.5)</td>
<td></td>
</tr>
<tr>
<td>yδ-1355</td>
<td>8</td>
<td>99</td>
<td>0.10 (0.3)</td>
<td>74.2 (74.9)</td>
<td>0.60 (0.1)</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate percent. LM, non-Tg littermates.

(data not shown) and, as the αβ T cells in normal mice (9), do not coexpress γδ TCRs at a detectable level (data not shown). These results indicate that a stringent mechanism for restricting the expression of TCR to one of the two types on a given thymocyte or splenic T cell is active in the Tg mice. Furthermore, the results suggest that in these Tg mice, and probably in normal mice, the γδ and αβ T-cell lineages branch out from common precursor T cells before either of the two types of TCR is expressed on the cell surface.

The γδ and δ Tg persist in the αβ T cells but their Transcripts Are Absent. To investigate why the Tg products are not expressed on the surface of αβ T cells, we purified αβ T cells (and, as a control, γδ T cells) from yδ-1355 mice by using a FACs and analyzed their DNA (Fig. 2A) and RNA (Fig. 2B).

The αβ T-cell population from γδ-1355 mice carries on average 1.1 copy per cell of Vγ4 gene segments in the germ-line configurations. Therefore, at most 0.9 (i.e., 2.0 copies minus 1.1 copies) copies per cell of the endogenous Vγ4 gene segment is expected to have rearranged. The observed 2.3 copies per cell of the VJγCγ γ gene (Fig. 2A) indicate that at least 1.4 (2.3 minus 0.9) copies per cell of VJγCγ γ can be attributed to the Tg. This is the minimum estimate of the Tg copy number because it is likely that some of the endogenous Vγ4 gene segments are deleted by rearrangement of the Jγ1-distal Vγ4 gene segment to the Jγ2 or Jγ3 gene segment (15). Thus, these results confirm that the γδ Tg is retained in the majority of αβ T cells.

Fig. 2A also shows the analysis of the δ Tg in the γδ-1355 mouse. The αβ T-cell population contains as many copies (about 4) of the Vγ4 gene segment rearranged to Jγ2 as does the γδ T-cell population, which clearly indicates that the δ Tg is retained in the αβ T-cell population. Analysis of DNA from γδ-1313 and γδ-1314 led to the same conclusion as to the retention of the Tg (data not shown).

\[ V_{γδ} \text{ and } V_{δ}^{γδ}-\text{specific RNAs were barely detectable in the } αβ \text{T cells purified from γδ-1355 splenocytes in contrast to the γδ T cells purified from the same mice (Fig. 2B).} \]

The same results were obtained with γδ-1313 and yδ-1314 (data not shown). We confirmed these findings at the level of cloned T cells: none of four randomly chosen αβ T-cell hybridomas prepared from γδ-1355 thymocytes, in contrast to two αβ T-cell hybridomas similarly prepared, contained RNA detectable with either the Vγ4 or Vδ T probe, despite their retention of the γδ Tg (data not shown). Thus, the expression of the Tg in the αβ T cells of the γδ Tg mice seems to be blocked at the level of RNA.

A Transcriptional Silencer in the Flanking Region of the γδ Gene. One possible interpretation of the absence of γδ transcripts in αβ T cells of the γδ Tg mice, and of normal mice (31), is that the γδ gene carries in its flanking region a cis-acting DNA element that down-regulates its own transcription in these cells. To test this possibility in vitro, we constructed another set of Tg mice by using a genomic DNA fragment, yγL (Fig. 1), that contains essentially the same γ gene as γδ-4 but much less flanking sequence. The copy numbers of the γδ Tg and the augmented levels of the γδ gene transcripts present in the thymocytes and splenocytes of three founder γδ Tg mice, yγL18, yγL18, and yγL19, are summarized in Table 1. The αβ T cells purified from the spleens of these Tg mice, in contrast to those from the γδ Tg mice (Fig. 2B), harbored abundant γδ RNA of an appropriate size [1.5 kilobases (kb)] detected by the Vγ4 probe (data not shown). Since the rearrangement of the endogenous Vγ4 γ gene was not inhibited in the αβ T cells of these Tg mice (data not shown), the Vγ4 probe-positive RNA may have been transcribed either from the γδ Tg or the endogenously rearranged Vγ4 γ gene or both. To investigate this issue, we prepared αβ T-cell hybridomas.

Fig. 2. Southern and Northern blot analyses of nucleic acids from the αβ TCR and γδ TCR splenic T-cell populations of the Tg mouse yδ-1355. (A) DNA extracted from the purified T-cell subpopulations as well as from C57BL/6 embryos (emb.) were digested with EcoRI and analyzed (5 μg per lane) with the Vγ4, Jγ2, or Thy1 probe. The band assignments were made according to previous studies (12, 20). gl designates the respective gene segment in the germ-line configuration. Vγ4 gl corresponds to an incompletely digested Vγ4 gene segment in the germ-line configuration (31). To deduce the copy numbers of the γδ genes and their fragments, the relative intensity obtained for each band was normalized for input DNA variation by using the intensity of the Thy1 band in the same lane. The copy number was then calculated by dividing the normalized relative intensity by that of the Vγ4 or Jγ2 germ-line band of the embryo DNA and then multiplying by 2. (B) RNAs extracted from the purified T-cell subpopulations were analyzed (10 μg per lane) with the Vγ4, Vδ5, or Cγ probe. The position of the 18S ribosomal RNA is indicated.
(and, as a control, γδ T hybridomas) from the thymocytes of the γ Tg mice γ817. As expected, each of these hybridomas, in contrast to the αβ T-cell hybridomas prepared from the γδ Tg mice, contained the Vγ4 probe-positive, 1.5-kb RNA (data not shown). Also as expected, most of these hybridomas contained, in addition to the γ Tg, an endogenously rearranged VJγ γ gene(s) and/or the Vαγ γ gene derived from the fusion partner BW5147 (15, 32) (Fig. 3A).

We prepared cDNA from total RNA extracted from the αβ T-cell hybridomas (as well as from the control γδ T hybridomas) and amplified a 200-base-long segment of the VγJγ γ-cDNA by the PCR (27) using appropriate oligonucleotide primers. The PCR products were incubated with the restriction enzyme Xho I and the reaction products were analyzed by agarose gel electrophoresis. Since the unique Xho I site is destroyed in the pγ-L Tg (Fig. 1), the amplified DNA corresponding to the Tg will be resistant to Xho I digestion, while any amplified DNA derived from endogenous or BW5147-derived VJγ γ genes will be split into 150- and 50-base-pair (bp) fragments. As shown in Fig. 3B, the three control γδ T-cell hybridomas (nos. 9, 11, and 12) yielded both 200- and 150-bp DNA fragments (the 50-bp DNA fragment, the other product of the Xho I digestion, was too short to be visualized clearly in the agarose gel). By contrast, all eight αβ T-cell hybridomas examined yielded only the 200-bp fragment indicative of transcripts from the γ Tg. Essentially the same results were obtained with the other two pγ-L-derived Tg mice, γ818 and γ819 (data not shown).

In conclusion, there exist abundant transcripts from the VJγ γ Tg in the T-cell hybridomas derived from the αβ thymocytes of the pγ-L Tg mice. In contrast, virtually no transcripts of the endogenously rearranged and/or BW5147-derived VJγ γ gene(s) are detected in the same T-cell hybridomas (Fig. 3B). Since these results were obtained with three Tg founder mice it is unlikely that the differential expression of the Tg and endogenous (and/or the thymoma derived) γ genes is caused by the specific sequence context in which the Tg are integrated. Thus, these results suggest that the γ gene carries, in its flanking regions, a cis-acting DNA element that down-regulates the amount of γ transcript in αβ T cells. The results further suggest that this silencer element is in the flanking regions present on the pγ-L clone but absent on the pγ-L clone.

**DISCUSSION**

Our finding that αβ T cells are generated in the γδ double Tg mice as abundantly as in non-Tg littermates does not support the Pardoll–Allison model. The αβ T cells retain the Tg but they are apparently transcriptionally silent. The analysis of Tg mice constructed with the short γ gene clone (pγ-L) strongly suggests that the transcription of VJγ γ genes present in αβ T cells is repressed via a DNA element (a silencer) located in its flanking region. In light of these findings we propose a model for the differentiation of αβ and γδ T cells (Fig. 4). The new model, like the Pardoll–Allison model, assumes that γ and δ gene rearrangements occur prior to the completion (i.e., V to DJ joining) of β gene rearrangement and the initiation of α gene rearrangement. Productive rearrangements of both γ and δ genes are, of course, a prerequisite for the generation of γδ thymocytes. However, the failure to rearrange both γ and δ genes productively is not a requirement for the generation of αβ thymocytes. We suggest that the putative machinery acting on the γ silencer is activated in a fraction of immature thymocytes and that it is from these cells that αβ thymocytes are generated. If the activation of the silencer machinery occurs prior to γ and δ gene rearrangements, αβ and γδ T-cell lineages are completely separate and no αβ thymocyte pre-

**Fig. 3.** Southern blot and PCR-aided Southern blot analyses of nucleic acids from T-cell hybridomas prepared from Tg mouse γ817. (A) DNAs extracted from eight (lanes 1–8) γ817-derived αβ T-cell hybridomas, six (lanes 9–14) γ817-derived γδ T-cell hybridomas, one (lane LM) normal mouse-derived αβ T-cell hybridoma, and the fusion partner thymoma BW5147 (lane BW) were digested with EcoRI and digested (5 μg per lane) with the Vγ4 probe. Since the Tg (10 copies) are concatenated, they appear as a heterogeneous band (Tg VγJγ Cγ) containing large (>30 kb) DNA fragments in contrast to the 16-kb band containing the endogenously rearranged and/or BW5147-derived Vαγ γ gene. Note that the lack of germ-line Vγ4 band (Vγ4 gl.) and/or the augmented intensity of rearranged Vαγ γ gene band of the non-Tg type (Endo Vαγ Jγ Cγ) over the intensity of the corresponding band of BW5147 DNA indicate that at least one copy of the endogenous Vγ4 alleles is rearranged in most hybridomas. (B) PCR products of RNA from the eight (lanes 1–8) αβ T-cell hybridomas and three (lanes 9, 11, and 12) γδ T-cell hybridomas, all derived from the Tg mouse γ817, were analyzed by Southern blotting with and without predigestion with Xho I. The 3′ Cγ probe used was the HindIII/Pvu II fragment (700 bp) from clone pγ-L (Fig. 1).
construct used, does not seem to be transcriptionally silent in an αβ T-cell clone (28).

Finally, the silencer model of T-cell development is supported by our recent finding that the generation of αβ T cells is severely blocked in Tg mice carrying γδ T Tg without the associated silencer (35).

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