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Models for Antigen Receptor Gene Rearrangement. II. Multiple Rearrangement in the TCR: Allelic Exclusion or Inclusion?[1]

Hannah Piper,* Samuel Litwin,[2†] and Ramit Mehr*

This series of papers addresses the effects of continuous Ag receptor gene rearrangement in lymphocytes on allelic exclusion. The previous paper discussed light chain gene rearrangement and receptor editing in B cells, and showed that these processes are ordered on three different levels. This order, combined with the constraints imposed by a strong negative selection, was shown to lead to effective allelic exclusion. In the present paper, we discuss rearrangement of TCR genes. In the TCR α-chain, allelic inclusion may be the rule rather than the exception. Several previous models, which attempted to explain experimental observations, such as the fractions of cells containing two productive TCRα rearrangements, did not sufficiently account for TCR gene organization, which limits secondary rearrangement, and for the effects of subsequent thymic selection. We present here a detailed, comprehensive computer simulation of TCR gene rearrangement, incorporating the interaction of this process with other aspects of lymphocyte development, including cell division, selection, cell death, and maturation. Our model shows how the observed fraction of T cells containing productive TCRα rearrangements on both alleles can be explained by the parameters of thymic selection imposed over a random rearrangement process. *The Journal of Immunology,* **1999, 163: 1799–1808.**

Multiple Rearrangements and Allelic Exclusion: A Contradiction?

Recent evidence shows that in the B cell receptor (BCR)[3] light chain (1, 2), or the TCR α-chain, rearrangement may not stop after a productive receptor gene has been formed and expressed. This raises the question: how is allelic exclusion maintained, if at all, in the face of continued rearrangement? The first paper in this series (41) showed, using computer simulation of BCR gene rearrangement, how continued light chain gene rearrangement can be reconciled with allelic exclusion. For $\alpha\beta$ T cells, the situation is more complex. As with the BCR heavy chain, allelic exclusion seems to be quite complete in the TCR β-chain (3–10). The expression of a functional TCR β-chain (in conjunction with a surrogate TCR α-chain (6)) triggers several successive cell divisions, which contributes to the shutdown of TCRβ gene rearrangement (7), followed by further differentiation (8) and the rearrangement of TCRα genes (9). Rearrangement and expression of TCR α-chain genes, on the other hand, does not stop after the expression of the first rearranged α-chain (3–5, 11–14). Rearrangement appears to continue until the cell is either positively selected, or dies (15, 16).

Due to the lack of allelic exclusion in TCRα, a T cell may not only contain two productively rearranged TCRα alleles, but also simultaneously express the two resulting TCRs. This is an alarming concept, because an $\alpha\beta$ T cell that matures in the thymus expressing two different TCRs may be positively selected on one of them, while the other TCR may be autoreactive (17). The frequency of T cells simultaneously expressing two different V_α genes was found in one study to vary between 10^{-3} and 10^{-4}. Only the cell surface expression of $V_\alpha 2$, $V_\alpha 12$, and $V_\alpha 24$ was monitored, which means that the frequency of T cells expressing any pair of V_α genes may be orders of magnitude higher (18). Independently, Malissen et al. (13) found that 26% of various T cell clones contained two productive V_α-J_α rearrangements (19).

The observations of allelic inclusion in TCRα raise the following questions. Can allelic inclusion be fully accounted for by multiple rearrangements alone? Do these rearrangements occur completely at random, or is there some underlying order? What is the role of positive and negative selection in driving, or limiting, the process of TCR gene rearrangement? Several models (reviewed below) were suggested in an attempt to answer the first question, but have not sufficiently addressed the issues of order in rearrangement and the role of selection. Here, we develop a model of the TCR gene rearrangement process, and use it to examine competing explanations for TCRα allelic inclusion. We aim to elucidate the mechanisms of allelic exclusion (or inclusion), and, in particular, to examine the degree of order in TCR gene rearrangement. Since the questions we study are probabilistic in nature, we use stochastic computer simulation of gene rearrangement and thymocyte selection. We perform simulations of our model under various parameter sets, and derive the constraints under which rearrangement and selection must operate (such as the average number of rearrangements performed per allele). Our results, briefly summarized, are: the $\alpha\beta$:$\gamma\delta$ ratio can largely be explained based on the number of cell divisions after β selection and thymic selection, but cannot be accounted for by rearrangement mechanisms alone. This is in contrast to the κ:λ ratio in B cells, which can be explained without invoking preferential expansion of κ B cells. The percent of TCR α "double-productive" T cells, on the other hand, is mainly determined by the probabilities of positive and negative thymic

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[3] Abbreviation used in this paper: BCR, B cell receptor.

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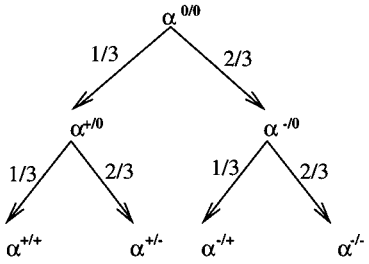

FIGURE 1. Malissen's model of the generation of α "double-expressors" (adapted from Ref. 13). The probability of a rearrangement being productive is at most one-third (this is the probability of joining in the correct reading frame), or slightly less if we take into account the existence of pseudo-genes. Thus, out of every nine cells, three cells would succeed in the first attempt to productively rearrange an α-chain gene, and, out of those, one will also productively rearrange the second allele; out of the remaining six cells, two will productively rearrange the second allele. (We denote the genotype of cells as follows: "0" denotes the unrearranged, germline configuration, a "+" denotes a productive rearrangement, and a "−" denotes a nonproductive rearrangement.) Three different genotypes will result: one cell will be $\alpha^{+/+}$ ("double-productive"), (2 + 2) cells will be $\alpha^{+/-}$, and four cells will be $\alpha^{-/-}$. The latter four will not survive selection, and hence the fraction of α "double-productives" among the cells that did survive thymic selection will be 1/5, or 20%.

selection, and the probabilities of resulting cell death. Death probabilities due to selection are smaller than death probabilities of developing B cells, which allow, on average, only two or three rearrangement attempts per cell, thus accounting for the apparent allelic exclusion in BCR chains. The presence of residual δ rearrangements in $\alpha\beta$ T cells (20–22) can be used to further delimit selection parameters. The fraction of productive residual δ rearrangements out of all residual δ rearrangements is found by all models, including ours, to be around 20% for a δ-first rearrangement pathway, in agreement with the experimental observations. This agreement supports the suggestion that TCRδ and γ rearrangement precedes α and β rearrangement.

In the following sections, we review previous models of TCR gene rearrangement, present our model and the results of computer simulations of this model, and conclude with a comparison of our findings for B and T lymphocyte gene rearrangement and development.

Review of Previous Models of TCR Rearrangement

TCRα rearrangements on both alleles

In this section, we review previous models of TCR rearrangement, on which our computer simulations rely. The value of 26% TCRα "double-expressors" found by Malissen et al. (19) was considered close to the value (20%) that one would expect if rearrangement of α alleles proceeded on both alleles, allowing only one rearrangement per chromosome (Fig. 1). However, Malissen's calculation did not take into account the possibility of multiple rearrangements on a single allele.

Mason (23), also allowing only one rearrangement per chromosome, additionally took into account the fact that the probability of a given T cell being selected to mature is very small. Under these assumptions, the fraction of α "double-expressors," $p(\alpha^{+/+})$, was calculated to be approximately half of the probability that a rearrangement is productive. If one assumes that this probability is $\sim$0.3, then $p(\alpha^{+/+})$ cannot exceed 15%. Mason's model is more realistic than Malissen's, yet the observed value of 26% is not compatible with its prediction (15% $\alpha^{+/+}$) for the case of only one rearrangement per allele. Thus, Mason further extended this model by allowing multiple rearrangements on each TCRα allele. The fraction of α "double-expressors" obtained, allowing a very large number of rearrangements per TCRα allele, is $p(\alpha^{+/+}) \sim 0.3$, which is close to the observed value of 26%. The fraction $p(\alpha^{+/+})$ decreases when the probability of a single rearrangement being positively selected increases, or when the number of rearrangements per cell decreases.

Our aim is to use a similar model to promote understanding of the mechanisms of rearrangement, addressing issues such as the average number of rearrangements performed per TCRα allele and the order (if any) in which they are performed. These factors could not be directly obtained from Mason's model, because it does not take into account the following two opposing constraints. First, Mason's model assumes rearrangement can continue ad infinitum, while, in reality, the numbers of V_α and J_α gene segments, though large, are not infinite. TCRα V-J rearrangements delete all gene segments between the two segments being joined (22), and hence, after several rearrangements, either the V or the J gene segment pool would be exhausted on that allele. Second, a mechanism that may partially compensate for gene segment pool exhaustion is order in gene rearrangement. This order refers to the apparent preference to rearrange first those J_α segments that are closer to the 5′ region of the J_α locus (10, 22, 24, 25). Additionally, we wanted to address the possibility of preference to rearrange the allele that was rearranged last, as suggested by studies on B cells (the first paper in this series). In T cells, TCRα rearrangement seems to go on simultaneously on both alleles (10, 13). However, weak preference for the most recently rearranged allele may still exist. In this study, we evaluate which of the two potentially opposing forces, the limited number of gene segments or the order in rearrangement, is more important in limiting TCRα rearrangement.

Mason's model lumps together the two nonpositive possible outcomes of the selection process: negative selection, or no selection (when the cell does not bind any self-MHC successfully, or the signals it receives are too weak for positive selection). These have to be addressed separately, due to their different effects on the probabilities of differentiation and death. The dependence of the outcome on the number, strength, and duration of signals the cell receives through its TCR is not yet fully known (26). This issue becomes more complicated when we consider that, if a cell expresses more than one receptor, the two receptors may be expressed with different cell surface densities (3). Our models do not directly address receptor expression; we assume that any productively rearranged gene is expressed at the maximum possible level and that the cell is selected according to the last rearrangement performed. However, our models deal with thymic selection through modifying the probabilities of the cell's death, maturation, cell division, or further rearrangement. A cell expressing an autoreactive TCR may receive strong negative selection signals, and, hence, survive for a shorter time (and thus be allowed fewer rearrangement attempts) than a cell expressing a receptor that does not bind any thymic MHC-peptide complexes. Hence, our models take into account the probability of intrathymic cell death as a function of the quality of the cell's TCR. The interplay between the strength of selection signals, and the potential for secondary TCRα gene rearrangement, will determine a cell's fate.

T cells may mature out of the thymus expressing a potentially autoreactive TCR, and the chance of this happening is probably higher for α "double-positive" T cells. There exists no experimental data indicating how many of the α "double-positive" T cells contain an autoreactive TCR, in addition to the TCR on which these cells were positively selected and allowed to mature. In the

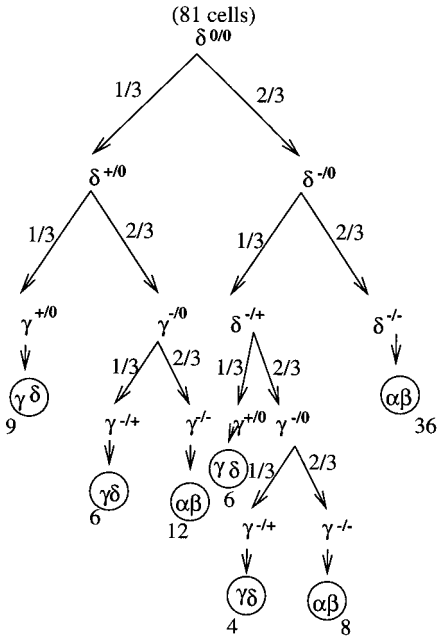

FIGURE 2. Hayday's model of the factors determining the $\alpha\beta$:$\gamma\delta$ T cell ratio. This model is strictly sequential and assumes no editing. Assuming strictly sequential rearrangement, cells that have failed to productively rearrange δ (4/9 of total) or γ (4/9 of the 5/9 that succeeded in δ rearrangement) proceed to the $\alpha\beta$ pathway (a total of 56/81 of the cells). Even if all cells that turned to the $\alpha\beta$ pathway had matured, the resulting ratio is ~2:1, an order of magnitude lower than the observed value; the difference was attributed to cell divisions in the $\alpha\beta$ lineage.

simulations presented below, it is easy to determine this value because we record the fate of every TCRα rearrangement.

The $\alpha\beta$:$\gamma\delta$ ratio and $\alpha\beta$ cells containing δ rearrangements

Any model of the TCR rearrangement process should also be able to account for the observed ratio of $\alpha\beta$ to $\gamma\delta$ T cells, observed to be 20:1 or larger, depending on the tissue being studied (27, 28). Not much is known about $\gamma\delta$ T cells, their function (29), development (30–32), or TCR γ- and δ-chain gene rearrangement. Most thymocytes try first to rearrange the δ-chain genes (22, 27), but this is not a rule (33); expression of TCRβ does not preclude differentiation into $\gamma\delta$ T cells (20, 34), and TCRβ and -γ transcripts can be detected simultaneously in the same cells (21).

Hayday and colleagues (20) suggested a model in which rearrangement is assumed to be strictly sequential (first δ, then γ, then β, and then α), and each allele can only be rearranged once. According to this model, 56 out of every 81 cells would end up in the $\alpha\beta$ lineage, after failing to productively rearrange a TCR δ-or γ-chain (Fig. 2). This results in an $\alpha\beta$:$\gamma\delta$ ratio of, at most, 2:1, 10-fold smaller than the observed ratio. The observed ratio must hence be explained as a result of subsequent cell proliferation in the $\alpha\beta$ T cell lineage. However, modifying this model to allow β rearrangements at any stage (before, during, or after δ or γ rearrangement), and multiple TCRα rearrangements, would reduce the final number of $\gamma\delta$ T cells produced. Thus, one question our models can be used to answer is: can the high ratio of $\alpha\beta$ to $\gamma\delta$ T cells be accounted for by assuming that rearrangement is not strictly sequential? Or do we have to also invoke multiple rearrangements on both α alleles to account for this high ratio?

Failure in rearrangement or expression of either γ- or δ-chain genes leads a cell to the $\alpha\beta$ pathway, yet the cell may still contain rearranged, perhaps even productively rearranged, γ and/or δ alleles. Indeed, $\alpha\beta$ T cells and thymocytes were found to retain up to 70–80% of the rearranged δ loci (22). However, all or most of these rearranged loci may exist on extrachromosomal DNA circles that were excised by the first TCRα rearrangement. Experimental measurements of the fraction of cells that have retained TCRδ genes within the TCRα locus on the chromosome would be extremely useful in determining the extent, and degree of order, of TCRα rearrangement. Our calculation, based on the same assumptions as the Hayday model (i.e., without multiple rearrangements), predicts that the fraction of $\alpha\beta$ cells that contain δ rearrangements will be 53.6%; and in those cells, 20% of δ rearrangements will be productive (Fig. 3). If, on the other hand, we extend this model to allow for multiple TCRα rearrangements, the fraction of $\alpha\beta$ cells that contain chromosomal δ rearrangements can be as high as 89% for the case of strict allele preference, depending on the number of rearrangement attempts per allele. If the exact value was experimentally measured, we could use our simulations (see below) to estimate the probability that a cell arrives at a productive TCRα rearrangement on one allele before starting to rearrange the other.

Rather more useful is the data on the fraction of residual δ rearrangements that are productive. According to all models, the fraction of productive (out of total) δ rearrangements is independent of the number of secondary rearrangements. This number is predicted to be 33% (the probability that a rearrangement is productive) in preselection thymocytes, but to decrease to 20% in $\alpha\beta$ thymocytes and T cells, because these subsets are depleted of cells that have succeeded to rearrange and express both δ and γ genes (Fig. 3). Indeed, the fraction of productive δ rearrangements was found to be between 17 and 24% in excised circular DNA $\alpha\beta$ T cells or thymocytes (20, 22) and as much as 29% in immature single-positive thymocytes (20). These observations may be used as an additional test for our simulation of TCR rearrangement.

A Simulation of TCR Gene Rearrangement

We constructed a stochastic simulation of TCR gene rearrangement.[4] A cell is "born" into the simulation and followed throughout its life in the thymus as it undergoes TCR gene rearrangement, cell divisions, and selection. Each cell of the final progeny is either allowed to mature or else dies intrathymically. This process is repeated for a large number of clones. The program is constructed of a number of modules, which correspond to the various processes the simulated cell undergoes, as follows.

1) Cell birth: a new cell is born; its TCR genes are all assigned the germline configuration.

2) Cell death: the cell is deleted from the simulation. Since thymocytes are thought to spend only ~3 wk in the thymus, cells that have not matured, but survived in the thymus up to the age of 20 days, die anyway.

3) Cell maturation: the cell's features are added to the accumulated statistics of T cells produced in the simulation, and it is deleted from the simulation.

4) Cell division: an additional copy of the current cell is produced (without changing the probabilities associated with the cell). The current cell's development is followed first, and the other daughter cell's development is followed next. This is a recursive process.

5) β-selection: after rearranging a productive TCR β-chain, the cell undergoes β-selection, that is, selection for the expression of a functional TCR β-chain; if the cell passes this obligatory step (with a probability $P_{\beta sel}$), it proceeds to rearrange the TCRα genes,

[4] The simulation program, and a program manual, containing a detailed description of the algorithm, are available from the authors upon request.

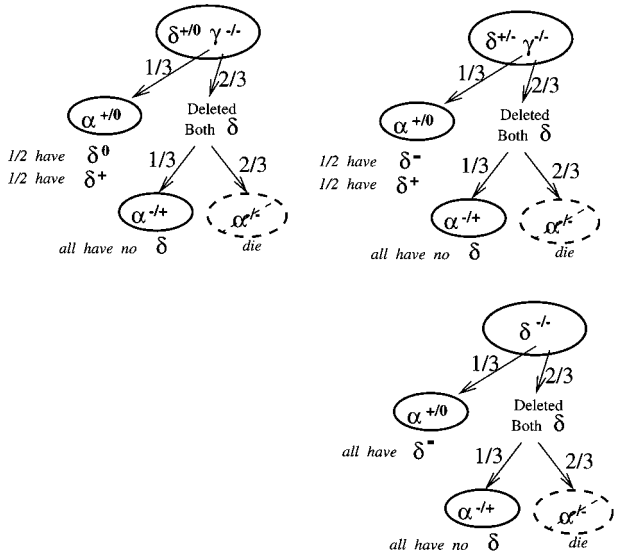

FIGURE 3. Our extension of Hayday's model. We calculate the number of productive and nonproductive δ rearrangements in αβ T cells. Calculations follow the same rules demonstrated in the previous figures; however, they were extended to trace the fate of TCRδ alleles in αβ cells. Out of the 56/81 cells that proceed to the αβ lineage, 20/81 or ~25% of the total number of cells will contain productive δ rearrangements, but rearrangement of the α locus may later excise the rearranged gene fragments. Fig. 3 thus shows that there are three starting points for cells going to the αβ lineage: TCR$\delta^{+/0}\gamma^{-/-}$, TCR$\delta^{-/+}\gamma^{-/-}$, and TCR$\delta^{-/-}$. The figure shows the calculation for each of these starting points. As we are only interested in percentages among mature αβ cells, we do not follow β rearrangement in this calculation because it does not affect the status of δ alleles. We only present the results of α rearrangement. For the 12/56 cells starting with a TCR$\delta^{+/0}$ genotype (*top left*), there is a probability of 1/3 that rearrangement of the first α allele will be productive. Out of these cells, 50% will have rearranged the α allele that contained the productively rearranged δ allele, and 50% will have rearranged the α allele that contained the nonrearranged δ allele. Hence, half of the cells with an $\alpha^{+/0}$ phenotype will contain a nonrearranged δ allele, and the other half will be left with a productively rearranged δ allele. Those cells that have failed to rearrange the first α allele but have succeeded with the second allele, ending up with an $\alpha^{-/+}$ phenotype, will have erased both δ alleles. Similar calculations were done for the 8/56 cells starting as TCR$\delta^{-/+}\gamma^{-/-}$ (*top right*) and the 36/56 cells starting as TCR$\delta^{-/-}$ (*bottom right*). When one adds up all the above combinations, the result is that 53.6% of all αβ cells retain a rearranged δ allele, and 20% of the retained δ rearrangements are productive. The above calculation does not include the possibility of multiple TCRα rearrangements. We can, however, derive an upper bound for the fraction of αβ cells that retain a rearranged δ allele, for the case of strict allele preference. This is done by replacing the probability of a rearrangement being productive (1/3) in the previous calculation, by P_1, the probability that the cell has reached a successful rearrangement on one allele only (possibly after a number of attempts), without rearranging the other allele. (The probability of failure, 2/3, is accordingly replaced by $(1 - P_1)$). Then, out of all cells going to the αβ pathway, the fraction of αβ cells with no δ allele surviving will be $P_1(1 - P_1)$; the fraction of αβ cells with an unrearranged δ allele surviving will be $6P_1/56$; the fraction of αβ cells with a nonproductively rearranged δ allele surviving will be $40P_1/56$; and the fraction of αβ cells with a productively rearranged δ allele surviving will be $10P_1/56$. The remaining $(1 - P_1)^2$ of the cells will die. Summing these numbers, the fraction of surviving αβ cells that contain δ rearrangements will now be $50/56(2 - P_1)$, which can be at most 89% (if $P_1 \sim 1$). It will be smaller if $P_1 < 1$, or if allele preference is not absolute, as shown in the simulations presented below. The fraction of productive (out of total) δ rearrangements is independent of the editing process and again equals 20%.

possibly performing a few cell divisions first (depending on the probability $P_{div\beta}$).

6) Thymic selection: operates on cells that have productively rearranged both TCRβ and TCRα. There are three possible outcomes: positive selection (with probability P_{+sel}), negative selection (P_{-sel}), or no selection (P_{0sel}). Positively selected cells may perform a few additional cell divisions before maturing (depending on the probability $P_{div\alpha\beta}$). Cells that were not positively selected may be allowed to perform additional attempts at rearrangement of TCRα, but death may occur before each attempt. Negatively selected cells are assigned a high probability of death.

7) TCRδ gene rearrangement: one of the TCRδ alleles is chosen and rearranged. If productive, the cell proceeds to rearrange a TCRγ gene. If not, it proceeds to rearrange the other TCRδ allele. If both failed, it proceeds to TCR β rearrangement (if TCR β alleles are still unrearranged).

8) TCRγ gene rearrangement: if productive, the cell matures as a γδ T cell. If not, it proceeds to rearrange the other TCRγ allele; if both failed, it proceeds to TCR β rearrangement. Secondary rearrangement, if allowed, occurs only once per allele.

9) TCRβ gene rearrangement: if it is productive, the cell proceeds to β-selection. If not, it proceeds to rearrange the other allele. If both failed, then the cell either goes back to δ (if this pathway was not tried earlier), or dies.

10) TCRα gene rearrangement: if productive, the cell proceeds to thymic selection. If not, it proceeds to rearrange the other allele, or rearrange the same allele when this is allowed. If both failed, the cell dies.

The simulation of the rearrangement process is similar to our model of BCR gene rearrangement (41), but is more elaborate, taking into account the rearrangement of all TCR chains, and all segments in each chain. One segment from each library (V, J, and, if applicable, D) is chosen at random. The probabilities for further rearrangements of the other segments are then renormalized, to account for deletion of intermediate segments. For example, if, at a given TCRα rearrangement, the simulation used V_{30} and J_{10}, then the probabilities of rearrangements for all V segments downstream of V_{30} and J segments upstream of J_{10} are set to zero, since we assume all rearrangements are deletional (22). The probabilities of choosing each of the remaining segments are assumed to be equal, unless we apply specific biases (see below). Rearrangement is deemed productive with probability $P_{product}$.

Parameters used in the simulations

Parameters for the simulations (shown in Table I) are interactively determined for each run. The values shown in Table I are those that we used as a "baseline," because they give a reasonable fit to most experimental observations (see below). $P_{product}$ was taken to be 0.33 in all simulations. The probabilities of cell division are discussed below. The probabilities of cell death at various stages are unknown and were varied in the simulations, as were the probabilities of rearrangement of δ vs β.

Two independent theoretical studies have estimated that about two-thirds of all receptors generated would be autoreactive (35, 36), and we used this value for P_{-sel}. Experimental studies on thymic selection have mostly been done with transgenic mice, in which all or most thymocytes undergo the same selection process, and hence cannot be used to estimate selection probabilities in a normal thymocyte repertoire. However, a study of mouse bone marrow chimeras estimated that one-half to two-thirds of thymocytes that underwent positive selection die before full maturation due to negative selection (37). As the fraction of thymocytes that actually mature, and hence have been positively selected, is very

Table I. *T cell simulation parameters with a sample of our "baseline" set of values*[a]

Parameter	Definition	Initial Value
N	Maximum number of cells to be simulated	100,000
N_l	Total number of lineages to be simulated	4,000
$P_{product}$	Prob. a rearrangement is productive	0.33
P_d	Initial Prob. of cell death	0.01
$P_{d\alpha}$	Prob. of cell death while rearranging α	0.1
P_{das}	Prob. of cell death if auto-reactive	0.7
P_{div}	Default Prob. of cell division	0
$P_{div\beta}$	Prob. of division after β-selection	0.5
$P_{div\alpha\beta}$	Prob. of division after thymic selection	0.4
$P_{\beta sel}$	Prob. of cell passing beta-selection	0.5
P_{-sel}	Prob. of cell being negatively selected	0.67
P_{+sel}	Prob. of cell being positively selected	0.03
$P_{R\delta 1}$	Prob. of δ1 rearrangement	0.495
$P_{R\delta 2}$	Prob. of δ2 rearrangement	0.495
$P_{R\gamma 1}$	Prob. of γ1 rearrangement	0
$P_{R\gamma 2}$	Prob. of γ2 rearrangement	0
$P_{R\beta 1}$	Prob. of β1 rearrangement	0
$P_{R\beta 2}$	Prob. of β2 rearrangement	0
$P_{R\alpha 1}$	Prob. of α1 rearrangement	0
$P_{R\alpha 2}$	Prob. of α2 rearrangement	0
E_γ	Multiple γ rearrangements allowed	False
E_α	Multiple α rearrangements allowed	True

[a] Once a cell has rearranged and expressed a $\gamma\delta$ TCR, it is allowed to mature; once a cell has rearranged and expressed an $\alpha\beta$ TCR, it undergoes thymic selection. Prob., probability.

small (~1–3%) (38), we use here the values $P(-sel) = 0.67$ and $P(0sel) = 0.30$.

Results

Interclonal variability and cell division

Preliminary simulations indicated that even 10,000 individual cells per simulation are insufficient, as demonstrated by the high variability between simulations that was observed (data not shown); with high cell division probabilities, 10,000 individual cells may all belong to a small number of clones. Thus, it was necessary to include a large number of independent clones (each possibly containing many cells) in each simulation, for parameters such as the $\alpha\beta$:$\gamma\delta$ ratio to stabilize. The number of clones was considered to be sufficient when both inter- and intrasimulation variabilities were small (<10% of the initial variability). This has been achieved for all quantities measured by generating 4000 independent clones in each simulation (data on variability not shown).

The above variability criterion helped to identify the proper division probabilities (probability of division following β-selection and following positive selection of $\alpha\beta$ T cells) to be used in the simulations. To understand what "division probabilities" mean in the model, we discuss what the simulated cell can do in each simulation step: it can either rearrange one of its TCR genes, go through a selection process, divide, or die, depending on the outcome of the previous step. Each of these operations takes a few hours in the real thymus, hence we think of our simulation steps as representing a time period of ~6 h (the minimum time required for cell division). Data shows that thymocytes do not usually perform more than one division per day on average (38). Specific measurements of cell divisions following β-selection suggest that cells passing this checkpoint perform about eight divisions in the course of 4 days (39). Later, cells that are positively selected may perform one or two additional divisions before leaving the thymus (40). Hence, in our simulations, values of $P_{div\beta}$ around 0.5, and lower values for $P_{div\alpha\beta}$, are reasonable.

Allowing cell divisions only following β-selection (up to a rate of $P_{div\beta} = 0.5$) had a very small effect on the $\alpha\beta$:$\gamma\delta$ ratio, because the cells still have to rearrange TCRα and pass thymic selection. On the other hand, simulation results are very sensitive to increases of the probability of division after positive selection. With $P_{div\beta} = 0.5$ and $P_{div\alpha\beta} = 0.25$, the $\alpha\beta$:$\gamma\delta$ ratio is only 2.5. However, the ratio increases quickly when we increase $P_{div\alpha\beta}$; the $\alpha\beta$:$\gamma\delta$ ratio is 4.7 when $P_{div\alpha\beta} = 0.4$, 27.0 when $P_{div\alpha\beta} = 0.5$, and as high as 200 when $P_{div\alpha\beta} = 0.6$ or higher (data not shown). Thus, values of $P_{div\beta}$, $P_{div\alpha\beta} \geq 0.5$ result in nonrealistic proliferation of positively selected cells. A simulation of 10,000 cells is largely taken over by one clone (even though we do not allow cells to remain in the simulated thymus for more than 80 simulation steps, or 20 days). The variability between simulations also becomes very large under these conditions, since individual clones may grow to high numbers so that each simulation represents a smaller number of thymocyte clones (data not shown). It is believed that positively selected cells do not perform more than a few divisions before maturing (40). Hence, in the following simulations the values of $P_{div\beta} = 0.5$, $P_{div\alpha\beta} = 0.4$ were used.

As long as proliferation is kept within reasonable limits, the total numbers of cells maturing from the thymus in the simulations remain small. Between 90 and 96% of the cells die intrathymically, as observed (38), which confirms our choice of selection probabilities ($P(-sel) = 0.67$ and $P(0sel) = 0.30$).

Preferential expansion must be invoked to account for the $\alpha\beta$:$\gamma\delta$ ratio

We proceeded to use the $\alpha\beta$:$\gamma\delta$ ratio as a way to identify the regions of parameter space that would give biologically reasonable results. We studied the dependence of this ratio on division probabilities, the probability $P_{R\delta}$ of starting with δ rearrangement, selection parameters, and death probabilities. The following results were obtained. First, as noted above, without cell divisions, or with small cell division probabilities, the ratio of $\alpha\beta$ to $\gamma\delta$ T cells remains small; it increases with division probabilities in the $\alpha\beta$ pathway. We did not consider the possibility of extensive cell death in the $\gamma\delta$ T cell lineage, because there is no evidence for such extensive death. Second, as expected, the highest ratio was obtained when it was mandatory to start with β rearrangement ($P_{R\beta 1} + P_{R\beta 2} + P_d = 1$, $P_{R\delta 1} = P_{R\delta 2} = 0$), so that the cell rearranges δ and γ only if it failed to productively rearrange a β-chain gene and express a functional β-chain. This case does not reflect the real dynamics and was used only to demonstrate the extreme limit. Third, the $\alpha\beta$:$\gamma\delta$ ratio is always higher when secondary α rearrangement is allowed, than in its absence. Obviously, if we allow secondary rearrangements in TCRγ as well, the $\alpha\beta$:$\gamma\delta$ ratio decreases (Fig. 4). However, multiple rearrangements in TCRγ did not significantly affect most results (data not shown), because there are only two Jγ segments, so secondary rearrangements can only occur once per γ allele. The most important insight was gained from simulations combining the above parameter variations: secondary rearrangements alone cannot give an $\alpha\beta$:$\gamma\delta$ ratio higher than 15, even in the extreme unrealistic case of a mandatory start with β rearrangement (Fig. 4). Cell divisions in the $\alpha\beta$ pathway thus have to be responsible for a large part of the $\alpha\beta$:$\gamma\delta$ ratio.

Random TCR gene rearrangement is compatible with TCRα allelic inclusion

Our main goal was to understand TCRα rearrangement; hence we studied the fate of productively rearranged TCRα alleles. We asked whether multiple rearrangements are necessary for reconstructing the experimental observations such as the fraction of α

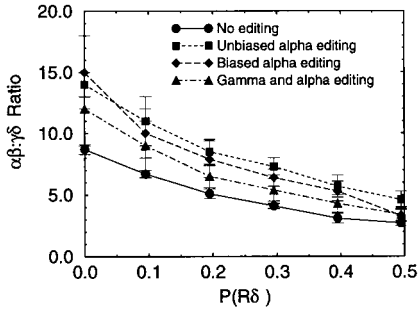

FIGURE 4. The $\alpha\beta$:$\gamma\delta$ T cell ratio. Ratios obtained in simulations as function of the probability of starting with rearrangement of a TCRδ allele. Since there are two such alleles, $P_{R\delta} = 0.495$ means that the probability of starting with a TCRδ rearrangement is $0.99 = 1 - P_d$, that is, starting with δ rearrangement is obligatory. "Biased editing" means we used $P_{allele} = P_{5'} = 1$.

"double positives" and the fractions of $\alpha\beta$ T cells containing residual δ rearrangements. To answer this, we studied the effects of changes in simulation parameters, especially the degree of order in rearrangement and the death probabilities, on results such as the fraction of α "double positives".

In each simulation, we recorded the number of $\alpha\beta$ T cells that have matured with both α alleles productively rearranged. In one series of simulations, $P_{d\alpha}$ was maintained at 0.1, while P_{das} was varied between 0.1 and 0.9. In another series of simulations, P_{das} was maintained at 0.7, while $P_{d\alpha}$ was increased from 0.1 to 0.9. In each series, simulations were performed without secondary α rearrangements or with biased or unbiased multiple α rearrangements. The latter terms can be explained as follows. There are two types of bias that can be applied to α rearrangements. The first type of bias is a preference to rearrange the same allele that was rearranged last. In our simulations, we used a parameter called P_{allele}, which took the value 0.5 if there was no preference, the value 1 if there was absolute preference to rearrange the last-rearranged allele (unless it was impossible), and the value 0 if there was absolute preference to rearrange the other allele. Intermediate values would mean partial preference; but as the effects of allele bias were not usually very large, we only used the values 0.5 or 1, that is, allele-unbiased or same allele-biased rearrangement. The second type of bias is a preference to use the 5′ J_α segments first. We used a parameter called $P_{5'}$, which took the value 0 if the choice of J_α segments was completely random, and 1 if the probabilities were biased. We only used a modest bias: when $P_{5'} = 1$, the probability of choosing the most 5′ J_α segment available is twice the average, and the probability of choosing the most 3′ J_α segment available is 0, while the probabilities of choosing intermediate segments changes linearly between the latter values. This still leaves some randomness in the choice of segments, and does not force a completely ordered rearrangement. Again, only the extreme cases of $P_{5'} = 0$ and $P_{5'} = 1$ were simulated, as the results fall between these extremes when intermediate values are used. When we refer to "biased rearrangement" or "biased receptor editing" without giving details, we mean that we used both types of bias, i.e., $P_{allele} = P_{5'} = 1$.

Not surprisingly, the percent of α "double productives" decreased with the increase of P_{das}, the death probability of "autoreactive" cells (Fig. 5). The effect on the percent of α "double productives" was not very strong, but it was consistent. In simulations performed with biased rearrangements, the fraction of α "double productives" was lower than the observed (and no higher than that obtained with no multiple rearrangements at all): it did not exceed 15% even for low values of P_{das} and/or $P_{d\alpha}$ (even when they were both set to 0; data not shown). Only when we simulated unbiased multiple rearrangements did we get higher fractions of α "double productives" (up to 25%). The conclusion from this result is that the experimental observations imply that α rearrangements are not biased, at least not as ordered as receptor editing in B cells seems to be. Furthermore, multiple unbiased rearrangements must be combined with a relatively weak negative selection to explain the observed 26% of α "double productives."

Is order in TCRα rearrangement masked by a large number of rearrangements per allele?

Our result on unbiased TCRα rearrangement agrees with the experimental measurement of α "double expressors," implying that there is no order in TCRα rearrangement. However, we cannot completely exclude the possibility that some small degree of order

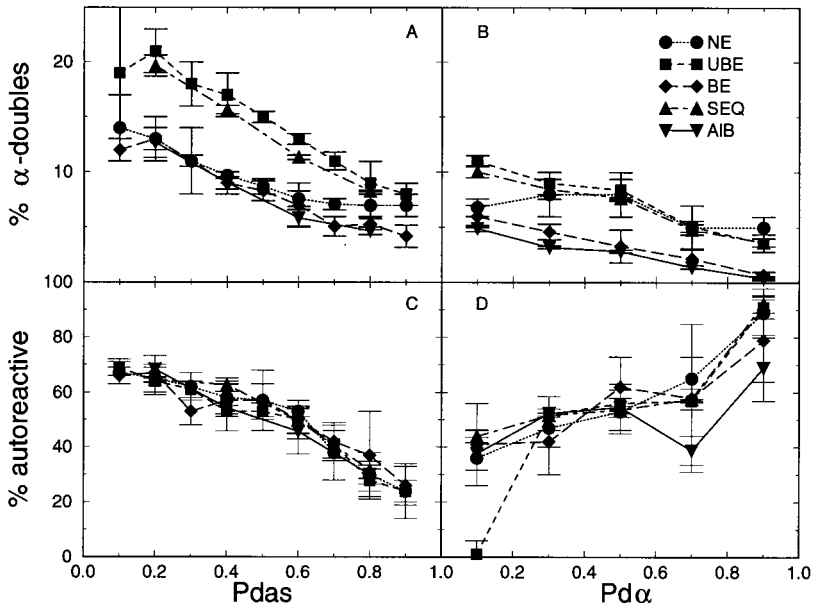

FIGURE 5. Effects of death probabilities. *A*, The fraction of α "double-productives" vs P_{das}. B, The fraction of α "double-productives" vs $P_{d\alpha}$. C, The fraction of "potentially autoreactive" cells within the α "double-productives," vs P_{das}. D, The fraction of "potentially autoreactive" cells within the α "double-productives," vs $P_{d\alpha}$. NE, no editing; UBE, unbiased editing; BE, biased editing (including both allele preference and sequentiality); SEQ, sequentiality only; AlB, allele bias only.

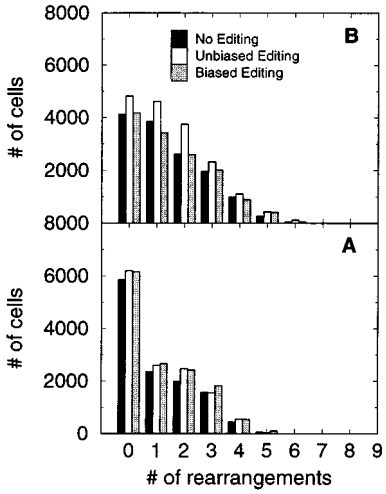

FIGURE 6. Rearrangements per allele. The distribution of number of rearrangements per TCRα allele in: A) a representative generic simulation with $P_{das} = 0.7$, and B) a simulation with $P_{das} = 0.2$, with all other parameter values remaining at default values (Table I).

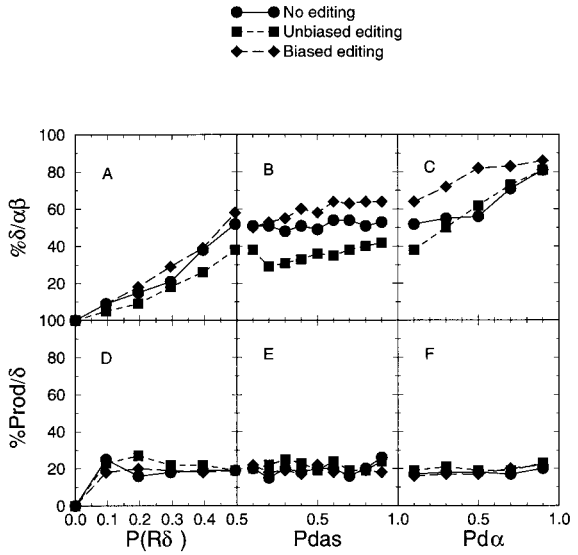

FIGURE 7. Residual TCRδ rearrangements in $\alpha\beta$ T cells. The fraction of $\alpha\beta$ cells that have a rearranged δ allele vs $P_{R\delta}$ (A), vs P_{das} (B), and vs $P_{d\alpha}$ (C). The percent of productively rearranged δ alleles, out of the rearranged δ alleles found in $\alpha\beta$ cells, vs $P_{R\delta}$ (D), vs P_{das} (E), and vs $P_{d\alpha}$ (F). Each point is an average of at least three simulations; error bars were not indicated, to make the figures clearer, but the typical variability was similar to that in Fig. 5.

does exist in the biological system, and is masked by a large number of rearrangements per cell. Even if P_{allele}, the probability for staying on a previously rearranged allele during a single rearrangement attempt is relatively high, the probability for staying on a previously rearranged allele after multiple rearrangements still decreases as the number of rearrangement attempts increases. To demonstrate this point, we show in Fig. 6 the distribution of the number of rearrangements per allele obtained in our simulations for the "default" parameter set (Table I) and unbiased rearrangement. This number was usually 3 or less, but in some cases was as high as 6, giving up to 12 rearrangements per cell. Even with only six rearrangements per cell, and with P_{allele} as high as 0.9, the probability of staying on a single allele throughout six rearrangements will be $(P_{allele})^5$, which is only ~0.59 for $P_{allele} = 0.9$, largely masking the inherent order.

Prediction: a potentially high fraction of TCRα double-expressors will carry an autoreactive receptor

One of the advantages of modeling is that it enables us to make predictions on quantities not previously measured in experiments. In the simulations described above, we have also counted the number of α "double positives" in which the second allele (that which did not result in positive selection and maturation of the cell) encoded an α-chain resulting in an autoreactive (vs nonselected) TCR. This is a worst-case estimate only, because it is obtained under the assumption that a cell is selected only according to its last rearrangement.

Theoretically, the fraction of "autoreactive α double-positives" should only depend on the last rearrangements on both alleles. The last of the two rearrangements will be the one that the cell was positively selected upon, but the previous one, being productive, must be either an anti-self rearrangement or a "neglected" one. Thus, we expect the fraction of cells expressing a "potentially autoreactive" TCRα allele to be at most $P(-sel)/[P(-sel) + P(0sel)]$, where $P(-sel)$ is the probability that a cell is negatively selected because its second to last rearrangement resulted in an autoreactive receptor, and $P(0sel)$ is the probability that a cell is neither negatively nor positively selected after its second to last (productive) rearrangement. With $P(-sel) = 0.67$ and $P(0sel) = 0.30$, the maximum fraction of "autoreactive" α "double-positives" will be 0.69. The effective value obviously decreases if we assign a larger death probability to negatively selected cells.

In the simulations, the fraction of "potentially autoreactives" decreased from the >60% predicted above for low P_{das}, to <30% with high P_{das} or $P_{d\alpha}$, which clearly shows the strong dependence on negative selection (Fig. 5). This result was independent of rearrangement order, as predicted by our analytical considerations above. It would be interesting to compare this value to experimental measurements, if and when these become available.

Residual TCRδ rearrangements in $\alpha\beta$ cells

We next considered the rearrangement status of TCRδ alleles in $\alpha\beta$ T cells. There can be at most one rearranged δ allele within the α locus on the chromosome in a mature $\alpha\beta$ T cell. Our analysis (Fig. 3) shows that, in a model of strictly ordered rearrangement (δ, γ, β, α), without multiple rearrangements, the fraction of $\alpha\beta$ cells containing a surviving rearranged δ allele within the α locus would be at most 53.6%. With multiple rearrangements, this value be as high as 89% (see legend to Fig. 3). However, this is only an upper bound, derived in the case of strict allele bias, and when $P_1 = 1$, P_1 being the probability that a cell will succeed in rearranging a nonautoreactive TCR α-chain gene on one allele only. Hence, we again turned to the simulation, recording the status of δ alleles, wherever there are undeleted δ alleles, in mature $\alpha\beta$ T cells. (When the rearranged δ allele is productive, the cell has become an $\alpha\beta$ T cell because no productive rearrangement of TCRγ was achieved on either allele.) In these simulations, values above 60% are only observed when there is order, and not with random rearrangement (Fig. 7). Thus, high fractions of $\alpha\beta$ T cells with chromosomal δ rearrangements imply some degree of order in TCRα rearrangement, although masked by the large number of rearrangements per cell. Since we have concluded above that high fractions of α "double productives" require a low degree of rearrangement order, it is unlikely that there are high percentages of $\alpha\beta$ T cells with chromosomal δ rearrangements. As the currently published

experimental measurements (22) do not clearly distinguish between chromosomal and extrachromosomal residual δ rearrangements, future measurements of residual δ rearrangements on the chromosomes will reveal further information on the degree of order in TCR gene rearrangement.

The fraction of αβ cells with a surviving rearranged δ allele increases with the probability of starting with δ rearrangement (Fig. 7*A*). It also increases when we increase either one of the death probabilities for αβ cells (Fig. 7, *B* and *C*). Again, this is because the smaller number of rearrangements per cell obtained for higher death probabilities may allow the order in rearrangement to be observed.

As an additional test of our simulations, we studied the fraction of productive δ rearrangements within the rearranged δ alleles. All analytical models predict that this fraction would converge to a value of 20%, which is within the range of experimentally observed values. Our simulations are also compatible with this observation, the value of 19 ± 3% is obtained. Even when the rearrangement process is not strictly sequential, we get similar results (Fig. 7, *C–E*).

Discussion

This paper is one of two studies that address the relationship between the allelic exclusion paradigm and the rapidly accumulating evidence for multiple rearrangements of Ag receptor genes in lymphocytes. The first study showed how allelic exclusion in B cells is maintained by the combination of ordered rearrangements and strong negative selection. Here, we extended our computer simulation of Ag receptor gene rearrangement to study rearrangement of TCR genes. We examined random vs ordered models of TCR gene rearrangement, and the interplay between this process and thymic selection. Our simulation takes into account stochastic rearrangement of the TCR variable region genes from their corresponding V(D)J gene segment libraries, several selection steps (β-selection; positive and negative selection of TCR-αβ-expressing cells), cell division, and cell death. The simulation follows each TCR clone from the start of rearrangement and records the fate of all daughter cells. We studied the properties of the emerging T cell repertoires under varying assumptions concerning the degree of order in the process of rearrangement. The main conclusions of the present study are the following. 1) High values of the αβ:γδ ratio cannot be obtained with multiple rearrangements alone; cell division must also be taken into account. 2) Multiple rearrangements of TCRα genes are most likely random, rather than ordered. 3) A high fraction of TCRα "double-productives" may express an autoreactive receptor. 4) The fraction of residual δ rearrangements in αβ T cells that are productive is ~20%, in agreement with experimental observations, thus confirming the accuracy of the analytical models. These conclusions are discussed in detail below.

The αβ:γδ ratio

One of the measurable quantities that has received much attention in the literature is the αβ:γδ ratio in thymocytes and mature T cells, which can be 20:1 or even higher, depending on the tissue studied. Theoretical predictions based on models that do not include multiple rearrangements fall around 2:1, which is far from the experimentally observed range of values. The difference was attributed to cell division. We set out to check to what extent multiple rearrangements may serve as an alternative explanation.

In our simulations, values compatible with the experimental observations were obtained only in the presence of multiple TCRα rearrangements. This is similar to our finding in B cells that the κ:λ ratio cannot be explained without multiple rearrangements. In contrast to the κ:λ ratio in B cells, however, high values of the αβ:γδ ratio cannot be obtained without also taking cell division into account. The ratio increases with the number of cell divisions after β selection and after thymic selection. Conversely, the ratio decreases when we increase the death probabilities of cells that fail β selection or αβ selection. Additionally, the αβ:γδ ratio increases with the probability that β rearrangement will precede δ rearrangement.

The fraction of TCRα "double-productives"

The measure for the extent of allelic exclusion, or rather allelic inclusion, in T cells, is the fraction of TCRα "double-productives," T cells that carry productive TCRα rearrangements on both alleles. Theoretical models predict that secondary rearrangements are necessary to explain the experimentally observed fraction of up to 26% TCRα "double-productives." Our simulations examined the dependence of this quantity on the degree of order in TCRα rearrangement and on selection probabilities. Fractions of TCRα double-productives higher than 20%, as observed experimentally, are obtained in our simulations only when we allow multiple TCRα rearrangements but assume they are unbiased, as in Mason's model. Thus, the results of these simulations cannot exclude the hypothesis that multiple rearrangements in T cells are random, rather than ordered as was found for the B cell light chain.

The fraction of autoreactive TCRα "double-productives"

A novel quantity defined in this study, for which no observations exist, is the fraction of cells with an autoreactive receptor among the TCRα double-productives. This fraction is independent of multiple rearrangements, because it depends only on the last rearrangements on the two alleles. However, we found that the fraction of autoreactive double-productives is highly sensitive to the death rate of autoreactive thymocytes. If this rate is low, as it must be to get 26% "double-productives," then the fraction of autoreactive double-productives can be as high as 70%. This value is only an upper bound, since it was obtained for the case in which the cell is selected only according to its last rearrangement. Otherwise, this number will be lower, and will also depend on the relative expression levels of the two receptors, which are not addressed by the current model. More experimental data would be beneficial for settling this issue, which may help elucidate instances of escape from central tolerance in T cells.

Residual δ rearrangements in αβ T cells.

The fraction of residual rearranged δ alleles in αβ T cells may also be helpful in revealing the details of the rearrangement process, due to the nesting of the δ locus within the α locus. The amount of residual TCRδ DNA in αβ T cells was observed experimentally to be as high as 80%; however, most of these rearranged alleles probably exist on extrachromosomal excised DNA circles (22). Our analysis of chromosomal δ rearrangements predicts that this value will very between 45 and 89%, depending on the parameters of TCRα editing. Our simulation confirms this prediction. Thus, these simulations can be used, in conjunction with future experimental measurements of the fraction of αβ T cells containing TCRδ rearrangements on chromosomes, to estimate currently unknown parameters, such as the death probability of unselected cells or the probability of rearranging δ before β. In our simulations, the fraction of rearranged δ alleles in αβ T cells increases when we increase the death probabilities of unselected or negatively selected

$\alpha\beta$-expressing thymocytes, because an increase in a death probability reduces the probability that the cell would rearrange both α alleles before maturing. The effect of the death probability of unselected cells is much more pronounced.

Among $\alpha\beta$ T cells that contain rearranged δ alleles, the fraction of these rearrangements that are productive was shown by all models to be independent of the number and order of rearrangements and predicted to be around 20%, which is within the range of experimental observations. Our simulations are consistent with this prediction under all conditions studied, confirming the accuracy of the analytical models.

TCR vs BCR gene rearrangement.

The first paper in this series (41) discussed rearrangement of the BCR light chain, for which isotypic exclusion and allelic exclusion have been established experimentally (1) in spite of observations on multiple rearrangement (reviewed in Ref. 2). Our computer simulation of BCR gene rearrangement enables us to reconcile multiple rearrangement with allelic exclusion. We provided evidence that, in B cells, 1) secondary rearrangements are negative-selection driven, in the sense that a cell has a limited time window in which it can edit its receptor and be rescued from deletion; and that 2) light chain rearrangement is an ordered process, on three levels: a preference for rearranging κ rather than λ light chain genes; a preference to make secondary rearrangements on the allele that has already been rearranged, rather than choosing the location of the next rearrangement at random; and, moreover, a sequentiality of rearrangement within each κ allele, such that J_κ1,2 are preferentially used before J_κ4,5. This order, combined with the stringency of negative selection, was shown to lead to effective allelic exclusion: the likelihood of a cell producing two productive rearrangements on two light chain alleles, within a limited time window and under the constraints of ordered rearrangement, becomes extremely small.

In spite of the strong similarities revealed in our studies between the way rearrangement seems to operate in B and T cells, it is worthwhile to note a crucial difference between the development of T cells to that of B cells. While BCR rearrangement seems to be limited by negative selection only, T cell development, on the other hand, seems to be limited by positive, rather than by negative, selection: developing T cells in the thymus are allowed a much more generous time window for continued TCR α rearrangement, so that multiple rearrangements on both alleles become the rule rather than the exception. Furthermore, while B cells generally exhibit allelic and isotypic exclusion, due in part to ordered rearrangement, in T cells, receptor gene rearrangement is far less ordered. As a result, the probability that a cell will contain more than one productive rearrangement of the TCR α-chain, and even express two TCR α-chains simultaneously, is far from negligible. Moreover, positive selection may rescue the cell from death, and allow it to mature, based on the virtues of only one of its expressed receptors, as long as the other receptor is not so extremely autoreactive as to cause immediate deletion of the cell. This is a potentially dangerous situation, because the second receptor may still be weakly autoreactive, or, worse, may be specific, with high affinity, to a self-peptide that is not presented in the thymus. In spite of the existence of peripheral mechanisms of self-tolerance, which safeguard against improper activation of T cell, such improper activation does sometimes happen. Thus, understanding TCR gene rearrangement, selection, and editing is key to understanding autoimmunity.

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