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Quantitation of Aberrant Interlocus T-Cell Receptor Rearrangements in Mouse Thymocytes and the Effect of the Herbicide 2,4-Dichlorophenoxyacetic Acid

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Small studies in human populations have suggested a correlation between the frequency of errors in antigen receptor gene assembly and lymphoid malignancy risk. In particular, agricultural workers exposed to pesticides have both an increased risk for lymphoma and an increased frequency of errors in antigen receptor gene assembly. In order to further investigate the potential of such errors to serve as a mechanistically based biomarker of lymphoid cancer risk, we have developed a sensitive PCR assay for quantifying errors of V(D)J recombination in the thymocytes of mice. This assay measures interlocus rearrangements between two T-cell receptor loci, V-gamma and J-beta, located on chromosomes 13 and 6, respectively. The baseline frequency in four strains of mice was determined at several ages (2–8 weeks of age) and was found to be stable at $\sim 1.5 \times 10^{-5}$ per thymocyte. Strain AKR, which has a high susceptibility to T-cell lymphomas, did not show an elevated frequency of aberrant V(D)J events. We used this assay to examine the effects of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) on the frequency of these events. Female B6C3F1 mice, 27 days of age, were exposed to 2,4-D by gavage at doses of 0, 3, 10, 30, and 100 mg/kg/day for 4 successive days and sacrificed on day 5. Thymus DNA was isolated and examined for illegitimate V(D)J recombination-mediated gene rearrangements. In addition, pregnant mice were exposed to 2,4-D and thymocytes from the offspring examined at 2 weeks of age. No significant increase in aberrant V(D)J rearrangements was found, indicating that under these conditions 2,4-D does not appear to effect this important mechanism of carcinogenesis. Environ. Mol. Mutagen. 42: 37–43, 2003.

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Key words: aberrant V(D)J recombination; mouse model; 2,4-D; cancer risk; biomarker

INTRODUCTION

Lymphoid malignancies are often characterized by chromosomal translocations that activate oncogenes. Many of these translocations are the result of mistakes made during the assembly of antigen receptor genes by V(D)J recombination [Tycko and Sklar, 1990]. In fact, ~35–50% of lymphoid malignancies contain translocations involving antigen receptor genes [Lieber, 1993]. Despite the necessity of V(D)J recombination for the generation of the antigen diversity needed for vertebrates to mount adequate immune defenses, this recombination process is inherently dangerous because of the DNA double-strand breaks generated with the accompanying potential for detrimental disorganization of the genome. Several laboratories have developed assays for measuring errors in V(D)J recombination as possible mechanistically based biomarkers of hematopoietic cancer risk, including recombinase-mediated deletions of the human *HPRT* gene [Fuscoe et al., 1991, 1997] and t(14;18) [Liu et al., 1994; Ji et al., 1995; Fuscoe et al., 1996]. In addition, Kirsch and colleagues [Lipkowitz et al., 1990; Kirsch et al., 1994] have developed useful assays for aberrant interlocus T-cell receptor (TCR) rearrangements in humans. These latter assays in particular have been used in small studies of human populations to show a correlation between the levels of TCR interlocus rearrangements in blood lymphocytes and lymphoid cancer risk [Lipkowitz et al., 1990, 1992; Abdallah et al., 1995]. Recently, Lista et al. [1997] have established a mouse model for measuring in-

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terlocus TCR rearrangements in thymocytes. This assay measures interlocus rearrangements between two T-cell receptor loci, TCRG variable (V) region and TCRB joining (J), located on chromosomes 13 and 6, respectively. In addition, assays were developed to measure the normal intralocus V-J rearrangements within the TCRG locus and within the TCRB locus. With this system it is now possible to adequately test hypotheses concerning the relationship between interlocus TCR rearrangements and hematopoietic cancer risk under controlled conditions. It is also possible to test chemical and physical agents for their ability to influence the fidelity of V(D)J recombination.

The assay described by Lista et al. [1997] uses radiolabeled probes to detect PCR amplified interlocus TCR rearrangement junctions. The frequency is calculated from the highest dilution of genomic DNA that gives a specific signal upon hybridization with the radiolabeled probe. Here we describe modifications of this assay which allow more precise quantitation of the rearrangements without the use of radiolabeled probes.

2,4-Dichlorophenoxyacetic acid (2,4-D) has been widely used for decades to selectively control broadleaf plants on lawns, roadways, forests, and agricultural land [IARC, 1986]. There have been numerous studies on the health effects of 2,4-D [IARC, 1986; Munro et al., 1992; EPA, 1994] and there has remained some controversy surrounding the potential linkage between exposure to 2,4-D and hematopoietic cancer, with little evidence from toxicology studies and suggestive evidence from epidemiology studies [Ibrahim et al., 1991]. Although there is little evidence of 2,4-D genotoxicity [Charles et al., 1999a,b; Gollapudi et al., 1999], Lipkowitz et al. [1992] found that the frequency of interlocus TCR rearrangements is increased in agriculture workers with environmental exposures to pesticides, including 2,4-D. Using our newly developed assay, we assessed the ability of 2,4-D to affect the frequency of interlocus TCR rearrangements under controlled conditions in the mouse.

MATERIALS AND METHODS

Mice and Chemical Exposures

Maintenance and treatment of all mice at the U.S. Environmental Protection Agency–Research Triangle Park animal facility conformed to institutional standards and all study protocols were approved by the Institutional Animal Care and Use Committee. All mice were given a diet of Purina rodent laboratory chow (Purina, St. Louis, MO) and water ad libitum. Pregnant female mice from four genetic backgrounds (Balb/c, AKR, C57BL/6, and C57BL/6 females mated with C3H males to obtain B6C3F1 pups) were obtained from Charles River Laboratories (Portage, MI) on day 14–16 of gestation. The resulting pups were randomly assigned to three groups and killed by asphyxiation with carbon dioxide at 13–15 days (2 weeks), 28–32 days (4 weeks), or ~60 days (8 weeks) of age. Approximately equal numbers of males and females were included in each group. B6C3F1 female mice were obtained from Charles River Laboratories at 21 days of age. The mice were randomly assigned to exposure and control groups and allowed to acclimate for 1 week. 2,4-Dichlorophenoxyacetic acid (2,4-D, CAS no. 94-75-7, Sigma, St. Louis, MO) was dissolved in dimethylsulfoxide at 500 mg/ml. This stock solution was then further diluted in corn oil so that administration of 0.2 cc to the animals resulted in doses of 100, 30, 10, 3, or 0 mg of 2,4-D per kg body weight. Animals were dosed by oral gavage on 4 successive days and were killed by asphyxiation with carbon dioxide on the fifth day. There were three mice per dose group. Additionally, timed-pregnant female C57BL/6 mice, mated with C3H males, were obtained at day 9 of gestation from Charles River Laboratories and randomly assigned to exposure and control groups. After a 5-day acclimation period, these mice were also treated with 2,4-D according to the previous dosing regimen on days 14–18 of gestation via oral gavage. The offspring were then killed by asphyxiation with carbon dioxide at 2 weeks of age.

The absorption, metabolism, distribution, and excretion of 2,4-D have been reviewed by Munro et al. [1992]. 2,4-D is absorbed quickly from the gastrointestinal tract (minutes to hours) and is distributed widely throughout the body. In addition, 2,4-D has been found in the uterus, placenta, fetus, and intrauterine fluid of rats and/or mice. It is almost entirely excreted, mostly in unaltered form, within 24 hr. Kavlock et al. [1987] have also shown that administration of 2,4-D to pregnant mice on days 8–12 of gestation resulted in significantly reduced birth weight. We therefore chose a dosing regimen that would expose thymocytes in adult mice to 2,4-D for 4 consecutive days and fetal thymocytes to 2,4-D during the developmental window when the thymus is first active (days 14–18).

Tissue and DNA Preparation

Thymuses from the study animals were removed, teased apart, and digested overnight at 55°C in digestion buffer (10 mM Tris pH 8.5, 25 mM EDTA, 100 mM NaCl, 1.5% n-lauroylsarcosine) with 0.7 mg/ml proteinase K. The digests were then sheared five times through a 21 gauge needle and incubated with 0.2 mg/ml RNase at 55°C for 1 hr. An additional 0.7 mg/ml of proteinase K was added, followed by at least another 1 hr of incubation at 55°C. The DNA was extracted three times using an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and finally dialyzed against TE (10 mM Tris HCl pH 8, 1 mM EDTA). DNA concentrations were measured spectrophotometrically. Several DNA concentrations were also determined or confirmed fluorometrically using Hoechst 33258 [Labarca and Paigen, 1980] and/or PicoGreen DNA dyes (Molecular Probes, Eugene, OR).

Detection of TCRG-TCRB Interlocus Rearrangements

A two-step nested PCR was used to detect the TCR rearrangements. The primary PCR reaction mix used to detect TCRG-TCRB (GB) translocations consisted of ~0.2–1 μg genomic DNA (representing 0.3–1.7 × 10^5 cells), PCR buffer (10 mM Tris pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin), 0.2 mM dNTPs, 0.31 μM each of outer primers A305 (5′-TCTACTCCAAACTACTCCAG-3′) and A308 (5′-ACCATACACTGGTACCGGCA-3′) [Lista et al., 1997], and 2.5 units Platinum Taq DNA Polymerase (Invitrogen, Grand Island, NY) in 30 μl total reaction volume. The secondary PCR reaction mix consisted of 1.2–2.0 μl of primary reaction products, PCR buffer (10 mM Tris pH 8.3, 50 mM KCl, 3.5 mM $MgCl_2$, 0.01% gelatin), 0.2 mM dNTPs, 0.31 μM each of inner primers A306 (5′-GGTGGAAGCGAGAGATGTGAA-3′) and A309 (5′-ACCCCTACCCATATTTTCTTAG-3′) [Lista et al., 1997], 2.5 units Taq polymerase (Promega, Madison, WI), and DNA loading dye (2.5% Ficoll, 0.005% xylene cyanole) in 30 μl total reaction volume. The use of the Platinum Taq DNA polymerase resulted in a hot start in which the polymerase is activated only after incubation at the denaturing temperature. The reactions were denatured at 94°C for 4 min, cycled 30 times through 94°C/15 sec; 55°C/15 sec; 72°C/60 sec, extended for an additional 5 min at 72°C, and finally incubated at 4°C. The secondary PCR reaction did not

TABLE I. Quantitation of TCR Rearrangements

Rearrangement[a]	Average no. of copies per PCR[b]	Observed P_0 (negative/total)[c]	Predicted P_0[d]	*P* value[e]
GB	1.0	0.40 (12/30)	0.37	0.71
BB	1.0	0.30 (9/30)	0.37	0.46
GG	1.2	0.23 (7/30)	0.30	0.55

[a]Intralocus rearrangement (BB and GG) and interlocus rearrangement (GB) junctions were cloned into pCR2.1 as described in Materials and Methods.
[b]PCR reactions were performed as described in Materials and Methods and included the indicated number of copies of linearized recombinant plasmids containing the cloned rearrangements. Genomic brain DNA from a male C57BL/6 mouse was also included in the reactions (2 μg, GB; 10 ng, BB; 10 pg, GG).
[c]P_0 is the fraction of PCRs with no detectable rearrangement. Numbers in parentheses show the number of null reactions/number of tested replicates.
[d]The predicted P_0 was calculated from the Poisson distribution with the indicated average number of rearranged molecules added to the reaction.
[e]Test of the null hypothesis that the observed fraction of negative PCRs is different from the predicted assuming a Poisson distribution with the mean equal to the average number of copies added per PCR.

employ hot start. Fifteen μl of the secondary reaction were loaded directly onto a 2% agarose gel. PCR products, visualized with ethidium bromide, indicated the presence of rearrangements. The position of the primers within the genes has been described previously [Lista et al., 1997].

Detection of TCRB and TCRG Intralocus Rearrangements

To detect TCRB (BB) rearrangements, ~0.2–0.5 ng of genomic DNA (representing 30–80 cells) along with outer primers A302 (5′-TGGTATCAACAGACTCAGGGG-3′) [Lista et al., 1997] and A305 were used in the primary reaction, and inner primers A303 (5′-TTCTCAGTCCAACAGTTTGAT-3′) [Lista et al., 1997] and A306 were used in the secondary reaction. For TCRG (GG) rearrangement detection, ~6–10 pg of template DNA (representing 1–2 cells) along with outer primers A308 and A311 (5′-TCATCACTGGAATAAAGCAG-3′) [Lista et al., 1997] were used in the primary reaction, and inner primers A309 and A312 (5′-GGTACTTACCGGAGGGAATT-3′) [Lista et al., 1997] were used in the secondary reaction. All other reaction conditions and cycling parameters were performed as indicated in the GB detection method. The position of the primers within the genes has been described previously [Lista et al., 1997].

For use as positive controls, GB, BB, and GG rearrangement PCR products generated from a C57BL/6 mouse thymus with the assays (and outer primers) described above were cloned into pCR2.1 (Invitrogen, Carlsbad, CA). After transformation into *E. coli* INVαF', the plasmids were amplified and purified using a Qiagen (Valencia, CA) midiprep kit. The plasmids were linearized with BamHI and quantified by spectrophotometry. The quantitation was confirmed by fluorometry using Hoechst 33258.

Quantitation of Rearrangement Frequencies

Rearrangement frequencies were calculated using a Poisson distribution model as previously described [Fuscoe et al., 1998]. Frequencies were estimated as -ln(1-p)/M, where p represents the fraction of PCR reactions containing at least one rearrangement and M is the approximate cell equivalents analyzed per PCR reaction. Ninety-five percent confidence intervals for each data point were also calculated as previously described [Fuscoe et al., 1998]. Briefly, the confidence limits were calculated by replacing p in the above formula with confidence limits for the fraction of replicates with at least one rearrangement, based on Mid-P exact confidence limits as described previously [Vollset, 1993].

DNA Sequence Analysis of TCR Rearrangement Junctions

PCR products were purified on Centricon 100 spin columns and quantitated by measuring the absorbance at 260 nm. Fifty-six to ninety ng of purified PCR products were then labeled using the ABI Prism Big Dye Terminator Cycle Sequence Kit and supplied protocol (Applied Biosystems, Foster City, CA) along with primers A306, A303, or A312 (for GB, BB, and GG, respectively) in a GeneAmp 9600 thermocycler (Applied Biosystems). Sequencing and analysis of products were then performed on an ABI 377 DNA Sequencer (Applied Biosystems).

RESULTS

In order to show that these PCR assays are capable of detecting a single copy of the TCR rearrangements, multiple replicate PCRs containing an average of one copy (BB and GB) or 1.2 copies (GG) were performed. The Poisson distribution was then used to predict the number of reactions that received no copies of the translocation DNA. The results are shown in Table I. Each of the assays was capable of detecting a single copy of the translocation, with no statistical difference between the expected frequency of translocations and the observed.

Using the TCR interlocus assay, we measured the background frequency of interlocus GB rearrangements in three strains of commonly used laboratory mice (B6C3F1, balb/c, and C57BL/6) at 2, 4, and 8 weeks of age. Twenty to thirty-five PCRs (average = 26) each containing 200–1,335 ng thymus DNA (average = 500 ng, representing 83,000 cell equivalents) from each of 10–22 mice (average = 14) were performed at each time point. The results are summarized in Figure 1. A two-factor ANOVA was used for analysis of these data. The frequencies at the various times within a given strain were not significantly different from each other. The data at the various times was therefore combined within each strain and a comparison was made between strains. No significant difference was found between any of these three strains. Figure 2 illustrates GB interlocus rearrangement PCR products following agarose gel electrophoresis. The DNA fragments were ~200 bp.

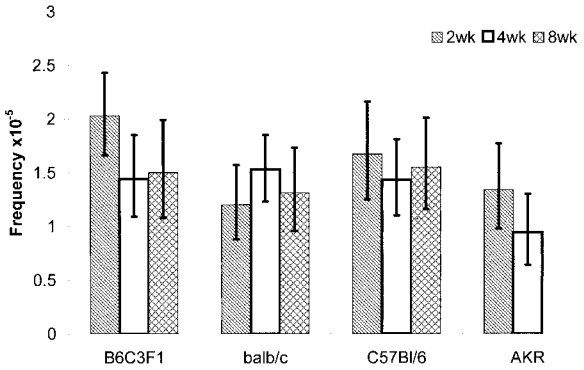

Fig. 1. Mouse thymus DNAs were examined for TCRGB interlocus rearrangements as described in Materials and Methods. The frequency and 95% confidence intervals for the population of mice in each group are presented.

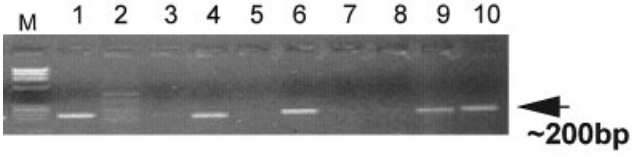

Fig. 2. Agarose gel electrophoresis of GB interlocus rearrangement-specific PCR products from the thymus DNA of a C57BL/6 mouse. Lanes 1–10 are from PCRs containing 1.0 μg DNA and performed as described in Materials and Methods. M, 200 ng ΦX174-HaeIII DNA.

DNA	*TCRGV3S1* Locus	N (or D)	*TCRBJ2* Locus
Germ Line	TCCTACGGCTAAAG		CTCCTATGAACAG-***TCRBJ2S7***
PCR GB-1	TCCTACGGCTAAAG	GGC	GAACAG-***TCRBJ2S7***
PCR GB-2	TCCTAC	TCCCCTGGGGGTATA	AACAG-***TCRBJ2S7***
PCR GB-3	TCCTAC	TCCCCTGGGGG	TATGAACAG-***TCRBJ2S7***
PCR GB-4	TCCTACGGC	CTT	CTATGAACAG-***TCRBJ2S7***
PCR GB-5	TC	GTG	GAACAG-***TCRBJ2S7***
PCR GB-6	TCC	CTCTCCCTTT	TGAACAG-***TCRBJ2S7***
Germ Line	TCCTACGGCTAAAG		AGTGCAGAAACGC-***TCRBJ2S3***
PCR GB-7	TCCTACGGCTA	GGACG	GTGCAGAAACGC-***TCRBJ2S3***
Germ Line	TCCTACGGCTAAAG		AGTCAAAACACCTTG-***TCRBJ2S4***
PCR GB-8	TCCTACGG	ACAACAGGACTGGGGGGG	TCAAAACACCTTG-***TCRBJ2S4***
PCR GB-9	TCCTACGG	TTACA	AAACACCTTG-***TCRBJ2S4***
Germ Line	TCCTACGGCTAAAG		AACCAAGACACCCAGTA-***TCRBJ2S5***
PCR GB-10	TCCTACGG	GG	AAGACACCCAGTA-***TCRBJ2S5***
DNA	***TCRGV3S1* Locus**	**N**	***TCRGJ1* Locus**
Germ Line	TCCTACGGCTAAAG		ATAGCTCGGGCTTT-***TCRGJ1S2***
PCR GG-1	TCCTACGGCT		ATAGCTCGGGCTTT-***TCRGJ1S2***
PCR GG-2	TCCTACGGCTAAAG		ATAGCTCGGGCTTT-***TCRGJ1S2***
PCR GG-3	TCCTACGG	CT**N**A**N**AGGGGGTAT	CTCAGGTTTT-***TCRGJ1S2***
DNA	***TCRBV5S1* Locus**	**N (or D)**	***TCRBJ2* Locus**
Germ Line	GTGCCAGCTCTCTC		CTCCTATGAACAG-***TCRBJ2S7***
PCR BB-1	GTGCCAGCTC	AACCGGGACACT	CTCCTATGAACAG-***TCRBJ2S7***
PCR BB-2	GTGCCAGCTCT	GCACA	CTATGAACAG-***TCRBJ2S7***
PCR BB-3	GTGCCAGCTCTC	GGACAGGGCTT	TATGAACAG-***TCRBJ2S7***
PCR BB-4	GTGCCAGCTCTC	GACCAA	TATGAACAG-***TCRBJ2S7***

Fig. 3. DNA sequence of TCR rearrangement junctions from the thymuses of 6-week-old C57BL/6 male mice. Germ line sequences are from Lista et al. [1997]. N, non-templated nucleotides. PCR products obtained with the GB assay are designated GB-1 to GB-10, those obtained with the GG assay are designated GG-1 to GG-3, and those obtained with the BB assay are designated BB-1 to BB-4. **N**, undetermined nucleotide.

DNA sequence analysis was used to confirm that the PCR products generated with these assays were indeed TCR rearrangements (Fig. 3). All PCR products examined from these assays (10/10 from the GB assay, 3/3 from the GG assay, and 4/4 from the BB assay) had the characteristics of true V(D)J recombinase-mediated TCR rearrangements, including cleavage at V(D)J recombinase recognition sequences, exonucleolytic "nibbling" of the joining ends, or the presence of N nucleotides.

The AKR inbred mouse strain is susceptible to spontaneous T-cell lymphoma arising in the thymus [see, for example, Haran-Ghera et al., 1995]. Figure 1 shows the frequency of TCR interlocus rearrangements in the thymus of this strain at 2 and 4 weeks of age. These data were added to the data from the other three strains and analyzed as described above. The only significant difference (P = 0.013) was found between the AKR strain (1.1×10^{-5}) and B6C3F1 (1.7×10^{-5}).

In addition, the frequency of intralocus TCR rearrangements was measured in the thymuses of the four strains of mice at 2 weeks of age. The frequencies per cell are given in Tables II (BB rearrangements) and III (GG rearrangements). There were no statistically significant differences in the levels of the specific normal rearrangements among the strains using ANOVA (P = 0.3 for the BB rearrangement and 0.16 for the GG rearrangement).

B6C3F1 adult female mice were exposed to 2,4-D at 0, 3, 10, 30, and 100 mg/kg as described in Materials and Methods for 4 successive days and sacrificed the following day. DNA was extracted from the thymuses and the frequency of GB interlocus rearrangements was determined. The experiment was repeated and the results of the two experiments were combined. The data are presented in Table IV. There was no evidence for a 2,4-D dose effect on the frequency of interlocus, aberrant TCR rearrangements under these conditions.

In order to assess the possible effects of 2,4-D on aberrant TCR rearrangements in the developing thymus, pregnant B6C3F1 mice were exposed on days 14–18 of gestation as described in Materials and Methods. The offspring were

TABLE II. Frequency of TCR BB Rearrangement Per Cell in 2-Week-Old Female Mice

Strain	Lower 95% confidence interval	Frequency	Upper 95% confidence interval
AKR	0.016	0.022	0.030
B6C3F1	0.014	0.020	0.027
Balb/c	0.012	0.017	0.023
C57BL/6	0.0093	0.014	0.021

TABLE III. Frequency of TCR GG Rearrangement Per Cell in 2-Week-Old Female Mice

Strain	Lower 95% confidence interval	Frequency	Upper 95% confidence interval
AKR	0.75	1.09	1.5
B6C3F1	0.67	1.00	1.39
Balb/c	0.63	0.91	1.22
C57BL/6	1.07	1.48	1.94

TABLE IV. Effect of 2,4-D on the Frequency of TCR GB Rearrangements in Adult B6C3F1 Female Mice

2,4-D dose (mg/kg)	Lower 95% confidence interval ($\times 10^{-5}$)	Frequency ($\times 10^{-5}$)	Upper 95% confidence interval ($\times 10^{-5}$)
0	1.51	2.51	5.83
3	2.19	2.91	4.22
10	1.56	2.03	2.80
30	1.58	2.02	2.76
100	1.44	2.09	3.36

TABLE V. Effect of 2,4-D on the Frequency of TCR GB Rearrangements in B6C3F1 Mice After Prenatal Exposure

2,4-D dose (mg/kg)	Lower 95% confidence interval ($\times 10^{-5}$)	Frequency ($\times 10^{-5}$)	Upper 95% confidence interval ($\times 10^{-5}$)
0	0.132	0.60	1.42
3	0.051	1.11	1.94
10	0.10	0.54	1.32
30	0.09	0.55	1.39
100	0.58	1.82	3.73

sacrificed at 2 weeks of age and the frequency of aberrant TCR GB rearrangements assessed. Two or three pregnant mice were treated with 2,4-D at all doses except 100 mg/kg; only one animal produced offspring at this dose. Three offspring from each treated dam were evaluated (except for two offspring from one of the 30 mg/kg group). The frequency of aberrant TCR GB interlocus rearrangements is shown in Table V. A one-sided trend test based on linear regression [Tukey et al., 1985] was conducted. There was no significant trend with increasing dose ($P = 0.08$).

DISCUSSION

A sensitive and specific PCR-based assay is described for the quantitation of aberrant interlocus T-cell receptor rearrangements in the thymocytes of mice. In addition, assays are described for the quantitation of normal intralocus rearrangements. Although these assays can be used to evaluate V(D)J recombination in any tissue, we chose to examine the thymus because this is the organ in which V(D)J recombination occurs and the frequency would not be influenced by the positive and negative selection processes that thymocytes undergo before they enter the general circulation. Thus, the fidelity of this critical process can be assessed. These assays are analogous to our previously described assays for the quantitation of V(D)J recombinase-mediated deletion of *HPRT* exons 2+3 [Fuscoe et al., 1997] and t(14;18) chromosomal translocations [Fuscoe et al., 1996] in peripheral blood cells of humans. Multiple replicate nested PCRs were performed on thymocyte DNA from individual mice and the presence or absence of rearrangement determined by agarose gel electrophoresis. The PCR conditions are robust and sensitive enough that Southern hybridization with radioactive probes is not required in order to detect the rearrangements. As described previously [Fuscoe et al., 1996], the Poisson relationship is used to calculate the frequency of aberrant rearrangements based on the fraction of PCR reactions that do not produce an assay-specific fragment. Reconstruction experiments show that the assays detect a single copy of the rearrangements. The specificity of the reactions was demonstrated by DNA sequence analysis of PCR products. All PCR products analyzed (10 from the GB assay, three from the GG assay, and four from the BB assay) showed the expected rearrangement. We have previously described statistical methodologies for this type of assay that can be used to compute confidence intervals for individual rearrangement frequencies [Fuscoe et al., 1996]. These statistical considerations enhance the sensitivity to detect differences between exposed and control populations.

Lista et al. [1997] were the first to describe assays for the assessment of the absolute number of the GB, GG, and BB rearrangements in mouse thymocytes. Using this assay, they showed that irradiation of newborn severe combined immune deficiency mice resulted in an increase in the levels of the GB transrearrangement that correlated with an increase in thymic lymphoma. Our assays make use of the primers described by Lista et al. [1997] and may provide some improvements. First, the specificity, sensitivity, and robustness of our assays remove the need for Southern analysis with radioactive probes. This saves time and avoids the potential hazards associated with the use of radionuclides. Second, we have developed statistical approaches for the analysis of the data that allow computation of confidence intervals on frequency estimates. This allows for robust testing of hypotheses using our assays. Finally, the new assays are also amenable to automation since they are based on simple PCRs.

Our results show that the frequency of aberrant interlocus rearrangements (GB) is remarkably stable in the thymocytes of three widely used strains of mice (B6C3F1, balb/c, and C57BL/6) during the 2–8-weeks of age development period (Fig. 1). We also found no significant difference in the rearrangement frequencies in 2- and 4-week-old AKR mice. Thus, during this 4- or 6-week time period the rearrangement frequencies do not change significantly. This stability may allow increased sensitivity for hypothesis testing be-

cause of the relatively low rearrangement frequency variability due to age.

In addition to the time-dependent stability of the aberrant GB rearrangement frequency within each strain, there is also a relatively constant frequency across strains. There was no significant difference in the frequency of the GB rearrangement in thymocytes between the B6C3F1, balb/c, and C57BL/6 strains ($\sim 1 \times 10^{-5}$). The frequency in the thymocytes from AKR mice was significantly lower than that from thymocytes from B6C3F1 mice and did not differ significantly from the other two strains. Thus, the error-prone component(s) of V(D)J recombination that generates these interlocus TCR rearrangements appears to be conserved in all four strains. There were also no significant differences in the frequencies of the normal BB and GG intralocus rearrangements among the strains at 2 weeks of age (Tables II, III). The frequencies of the normal BB and GG rearrangements were ~1 per 100 thymocytes and 1 per thymocyte, respectively, which is similar to the frequency found by Lista et al. [1997].

AKR mice spontaneously form thymic lymphomas at a high frequency (>90%) and this has been shown to be due to endogenous, genetically transmitted viruses [Rowe, 1972; Fischinger et al., 1975; Hartley et al., 1977; Chattopadhyay et al., 1980]. We tested the hypothesis that increased levels of aberrant V(D)J recombination played a role in the increased sensitivity of the AKR strain to T-cell lymphoma. Our finding that the frequency of aberrant V(D)J recombination, as reflected in the levels of interlocus TCR rearrangements, is not significantly different from strains without this high level of lymphoma suggests that this potential mechanism of tumorigenesis is not a factor in the etiology of these tumors. In fact, it appears that the sensitivity of the AKR strain to T-cell lymphomagenesis is the direct result of endogenous and recombinant viral proteins, since antibodies to these proteins suppressed the frequency of spontaneous tumors [Haran-Ghera et al., 1995].

Because of the reported epidemiological suggestions of an association between exposure to 2,4-D and hematopoietic cancer [Hoar et al., 1986; Zahm et al., 1990], there has remained some concern over the potential carcinogenic effects of this herbicide, despite little evidence from toxicology studies [Ibrahim et al., 1991; Charles et al., 1996]. Interestingly, Garry, Kirsch, and co-workers [Lipkowitz et al., 1992; Garry et al., 2001] found evidence that aberrant V(D)J recombination occurs in peripheral blood lymphocytes of agriculture workers with exposures to pesticides, including 2,4-D, suggesting a mechanism of genomic instability by which tumors may arise. Using our newly developed assay for the quantitation of the analogous aberrations in mice, we tested the hypothesis that exposure to 2,4-D would increase the levels of aberrant V(D)J recombination as reflected in interlocus TCR rearrangements. Two experiments were carried out. The first was on 4-week-old female mice that were given 2,4-D by daily gavage for 4 successive days and then sacrificed on the following day. Doses were chosen to cover a wide range of exposures, with the highest dose being about one-third of the LD_{50} [IARC, 1977] and the lowest being about 1% of the LD_{50}. Under these experimental conditions, no significant increase in the frequency of interlocus TCR rearrangements was observed, suggesting that 2,4-D does not effect fidelity of V(D)J recombination in adult mice and lead to genomic instability.

In the second experiment, we assessed the ability of 2,4-D to cause these aberrant rearrangements in the developing thymus. Pregnant C57BL/6 mice were exposed to 2,4-D by gavage on days 14–18 of gestation and thymocytes from the pups were examined at 2 weeks of age for aberrant interlocus TCR rearrangements. Again, the doses spanned a wide range, approaching one-third of the LD_{50} dose. No statistically significant increase in the frequency of the aberrant rearrangements was found. Thus, under these conditions 2,4-D does not appear to effect this important mechanism of carcinogenesis.

Chromosomal translocations mediated by aberrant V(D)J recombination are a major mechanism for activation of oncogenes in hematopoietic tumors [Aplan et al., 1990; Brown et al., 1990; Croce, 1993; Rabbitts, 1994]. In addition, it has been reported that illegitimate V(D)J recombination was responsible for inactivation of tumor suppressor genes in lymphoid leukemia [Cayuela et al., 1997; Kitagawa et al., 2002]. Until recently, however, a model system has not been available for the controlled testing of medical, occupational, and environmental chemicals to influence the fidelity of this critical process [Lista et al., 1997]. The improvements to this in vivo mouse assay described here should allow the efficient evaluation of chemicals for this property.

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