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Allelic exclusion in rat kappa immunoglobulin chains: extent of J_k rearrangement in normal B lymphocytes

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The frequency of normal rat peripheral B lymphocytes stained for surface immunoglobulin kappa allotypes *a* and *b* in (a x b) F_1 heterozygotes was assessed by two-colour immunofluorescence on a fluorescence-activated cell sorter. The upper limit to the frequency of double-stainers was 8% among all kappa-positive cells, though it was not resolved how far cytophilic antibody accounted for these. Cells expressing each allotype singly were isolated and the extent of rearrangement of the genes encoding the joining-kappa segment on the expressed and non-expressed chromosome were independently assessed. The expressed allele was found to be virtually completely rearranged while the non-expressed allele showed ~45–60% rearrangement. The implications of this substantial non-productive rearrangement for models of allelic exclusion are discussed.

Key words: allelic exclusion /B lymphocytes/immunoglobulin kappa gene rearrangement/J_k RFLP

Introduction

B lymphocytes of the peripheral lymphoid system have reached the stage of differentiation where their surface immunoglobulin awaits the trigger of antigen to complete their development into antibody-secreting cells. The ability of a B cell to express only one of an allelic pair of immunoglobulin light and heavy chains is termed allelic exclusion (Pernis *et al.*, 1965; Weiler, 1965; Pernis *et al.*, 1970), the molecular mechanism of which is unknown. Synthesis of immunoglobulin first requires the translocation to a specific site of a variable (V) gene segment to a functional (non-pseudogene) joining (J) segment thus eliding the variable and constant (C) regions into a single transcriptional unit. In the case of heavy chains, which rearrange before light chains in ontogeny (Alt *et al.*, 1981; Maki *et al.*, 1980; Perry *et al.*, 1981; Joho *et al.*, 1983; Coffman and Weissman, 1983), this process is embroidered by the incorporation of a short 'diversity' gene segment but there is no reason otherwise to think that heavy and light chain rearrangement differ fundamentally.

The simplest explanation for allelic exclusion would be that a V gene segment translocates to a J gene segment on only one of the two homologous chromosomes, leading to its subsequent expression ('restricted rearrangement') (Joho and Weissman, 1980; Early and Hood, 1981). The other chromosome which has not undergone V/J translocation would remain silent, maintaining its germ-line configuration. An alternative hypothesis postulates that the expression of a particular allele is determined by an essentially random process governed by the frequency of translocation and by the relative probability of occurrence of a productive *versus* a non-productive V/J rearrangement (a 'probabilistic' model (Coleclough *et al.*, 1981; Altenburger *et al.*, 1980; Coleclough, 1983). This model allows, indeed predicts, a certain amount of rearrangement of the non-expressed chromosome; failure to be expressed would result from aberrant rearrangements (wrong reading frame, creation of a termination codon or use of pseudo-J gene, for example).

In the case of heavy chains, studies of hybridoma and myeloma cell lines (Cory and Adams, 1980; Coleclough *et al.*, 1980) and of normal peripheral B cells (Nottenburg and Weissman, 1981) showed that nearly all the germ-line context is lost; the extent of rearrangement is much greater than is needed to account for the expressed chromosome alone. For light chains, antibody-secreting cell-lines show many examples of rearrangements of both chromosomes (Altenburger *et al.*, 1980; Perry *et al.*, 1980; Coleclough *et al.*, 1981); however, in a study of kappa-positive lymphocytes from normal mouse spleen only about half the kappa DNA was rearranged (Joho and Weissman, 1980), which was taken as evidence that V_k/J_k joining occurs for the most part on only one chromosome of the majority of kappa expressing B lymphocytes. When Coleclough *et al.* (1981) and Van Ness *et al.* (1982) performed a more detailed analysis they found that 65% of the kappa DNA was rearranged as judged by the loss of the germ-line J_k-containing restriction fragment relative to the C_k fragment. However, because no allotypes of mouse kappa have been found, rearrangement of the non-expressed chromosome could not be examined directly. The interpretation of these data on normal B cells rested on the assumption of virtually complete allelic exclusion since double-cells, if they existed, would be expected to have rearranged both chromosomes. For kappa chains allelic exclusion has been most exactingly tested in the rabbit (Pernis *et al.*, 1970; Jones *et al.*, 1973; Loor and Kelus, 1978) and in this case the allotypes very often are expressed in unequal proportions ('allelic preference'). This might mask some double-expression, which would have been more obvious if the alleles were equally represented. Some doubt therefore remains whether the degree of rearrangement is so great that it must necessarily extend to the non-expressed chromosome.

The opportunity presents itself in the rat to test directly the extent of rearrangement of the non-expressed kappa chromosome. Rats make two serologically-defined constant region kappa allotypes (Gutman and Weissman, 1971), our prototype inbred strains for which are DA *(Igk-1^b)* and LEW *(Igk-1^a)*. The congenic line LEW-*Igk-1^b* has been founded by 10 generations of backcrossing followed by extraction of the homozygote incross (Rozing *et al.*, 1979). We undertook two studies of heterozygotes, either (LEW x DA)F_1 or (LEW x LEW-*Igk-1^b*)F_1. By two-colour fluorescence analysis on a fluorescence-activated cell sorter (FACS) we examined how

rigorously allelic exclusion operates for surface kappa chains of peripheral (thoracic duct or lymph node) B lymphocytes. We then prepared DNA from cells expressing one or other allotype in these heterozygotes and used a dimorphism of the length of the J_k-containing fragment generated by the restriction enzyme *PstI* to distinguish the germ-line *a* from the *b* chromosome, assaying rearrangement by the disappearance of the fragment in Southern blots (Joho and Weissman, 1980).

Results

Two colour fluorescence analysis of surface kappa allotype expression in heterozygotes

Thoracic duct lymphocytes from (DA x LEW)F_1 rats were centrifuged over a metrizoate-Ficoll gradient to remove dead cells and washed three times before staining with a three-stage procedure to detect Igk-1^a (indirect fluorescence with 'Texas Red') and Igk-1^b (direct fluorescence with fluorescein). The first layer was biotin MAR80 IgG (anti Igk-1^a), then Texas Red-avidin and finally fluorescein-conjugated anti-Igk-1^b. As a further precaution to examine only viable cells, propidium iodide was included at the end of the final incubation and cells brightly stained with this vital dye were electronically gated out from the FACS analysis (<1% of total cells) (Parks *et al.*, 1983). Since the anti-Ig-1^b was an alloantibody, itself of *a* allotype, care was taken to prevent interaction between the staining antibodies by including a 5-fold molar excess of IgG^a (200 μg/ml) in the second and third stage reagents. To judge the effectiveness of this inhibition and to control for cytophilic antibody, thoracic duct lymphocytes from a radiation chimera constructed by injecting homozygous Igk-1^a bone marrow into Igk-1^b recipients were stained in parallel with the F_1 sample.

Contour plots of the frequency of red and green staining cells among a sample of 100 000 are shown for one experiment out of three performed (Figure 1a). Four populations could be distinguished, demarcated by valleys in the frequen-

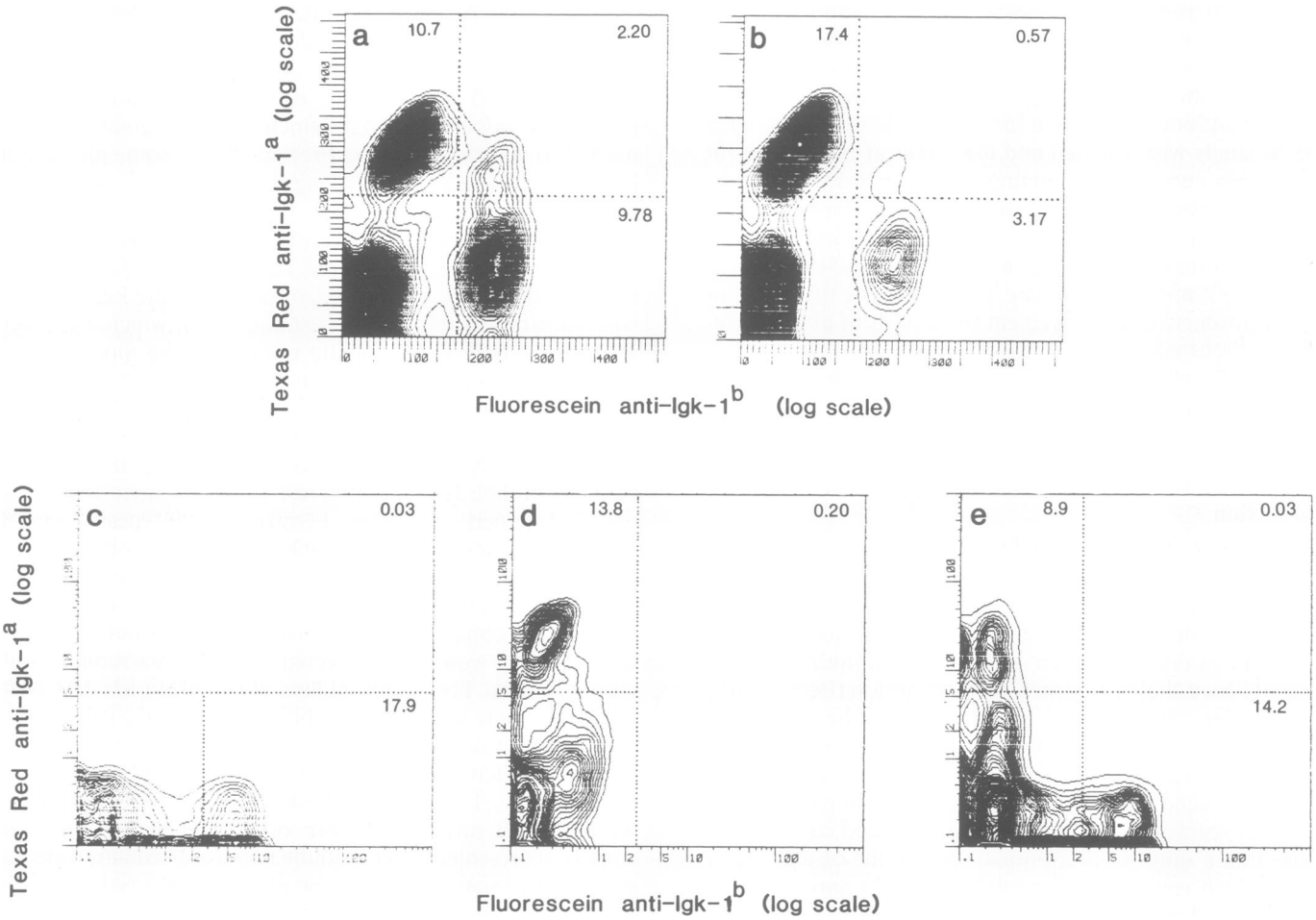

Fig. 1. Two colour FACS analysis of 10^5 thoracic duct lymphocytes **(a,b)** or lymph node lymphocytes **(c,d,e)**, gated to exclude dead cells (1% and 5% of total, respectively) by low angle light scatter and propidium iodide staining. For **a**–**d** staining was in three stages: (i) biotin-MAR 80 (anti-Igk-1^a). (ii) 'Texas Red'-avidin. (iii) fluorescein-anti-Igk-1^b. **(a)** (LEW x DA)F_1; **(b)** LEW LEW-*Igk-1^b* chimera; **(c)** (LEW x DA)F_1, including 200 μg/ml Igk-1^a IgG in first stage; **(d)** (LEW x DA)F_1, including 200 μg/ml *Igk-1^b* IgG in third stage; **(e)** (LEW x DA)F_1, staining 2 aliquots separately for each antibody singly, then mixed in equal numbers to check cross-talk between FACS channels; figures indicate percentage among all cells falling within stated windows. In two further experiments on (LEW x DA)F_1 cells [cf, **(a)**], the percentages were: a^-b^+, 13.3, 14.0; a^+b^-, 10.9, 10.7; a^+b^+, 1.7, 2.3. Converting all the data to percentages among Ig^+ cells gives:

	(LEW x DA)F_1			Chimera
Expt.	1	2	3	3
a^-b^+	52	51	43	15
a^+b^-	42	40	47	82
a^+b^+	6.6	8.5	9.7	2.7

cy distribution. These corresponded to unstained cells (presumably T cells), red-only cells, green-only cells and a small but distinct group of double-stainers. The legend to Figure 1 summarises the frequencies of these four populations. Overall (though not in the particular experiment illustrated) Igk-1^b-positive cells predominated slightly over Igk-1^a-positives (mean 49% against 43% among B cells) as expected from earlier studies of allelic preference (Nezlin and Rokhlin, 1976; Hunt and Williams, 1974). In both the F_1 and the chimera the single expressors exhibited very low levels of the other allotype, which at least in the chimera can only have been passively acquired: for instance, the cells stained red were also slightly green, to a greater extent than the T cells. This was not due to a technical artefact due, for example, to interaction between the staining antibodies or to cross-talk between the channels of the cell sorter, since it was not seen when homozygous cells were stained (Figure 1). This degree of cytophilic antibody was clearly distinguishable by a trough of fluorescence intensity from the levels of expression seen with the small group of double-stainers (mean frequency 8.3% among F_1 B cells), which are the cells carrying substantial amounts of both allotypes. For both double and single expressors the distinction between labeled and unlabeled was clearer for the fluorescein fluorescence (one stage staining) than for the Texas Red (two stage) so no particular significance is attached to the fact that the island of double-positives seems to fuse more with the green-only group than the red-only group: the modal level of expression of either allotype on double expressors is not greatly different from that on single expressors. The broad conclusion from these results is therefore that in the heterozygote most immunoglobulin-bearing cells (presumptive B cells) carry a single allotype, with a slightly higher frequency of Igk-1^b expressors than Igk-1^a, but that there is a small fraction of cells, $<10\%$ of all B cells, which carry both.

The question arises whether the double-stainers are truly synthesising their surface Ig, or whether it is merely cytophilic for a minor population of lymphocytes, which could be either T or B. The observation that in the chimera $<3\%$ of B cells stain both red and green suggests that not all can be attributed to cytophilic antibody in the F_1 where the frequency was higher, 8%. This assumes that a chimera 6 months after reconstitution contains a thoracic duct population like that of a normal unirradiated rat. In respect of gross characteristics such as thoracic duct hourly output of lymphocytes and B/T ratios they are very similar but the possibility remains that the lower frequency of double stainers in the chimera is attributable to incomplete reconstitution after irradiation. The chimera also differed from the F_1 in the ratio of Ig-1^a to Ig-1^b ($\sim$6 to 1) but it is not likely that this imbalance would cause a deficit of Igk-1^b-bearing double stainers since plasma Ig concentrations are probably in considerable excess of the concentration needed to stick to cells. So with these provisos we concluded that at least some of the double-staining cells in the F_1 have made both allotypes themselves and their DNA would be expected to be rearranged fully on both chromosomes. For the smaller proportion whose Ig seems to be cytophilic then their DNA might be partly or not at all rearranged, depending on whether these cells were or were not B lymphocytes.

Identification of the two allelic chromosomes by their restriction fragments

To examine the context of the J_k segment on each of the two chromosomes of a heterozygote it was necessary to find an allotype-related size polymorphism of a restriction fragment of DNA containing the segment. Ideally a restriction endonuclease was needed which would cleave the J_k-containing segment from the C_k fragment. The C_k-containing fragment would then retain its germ-line context in mature B cells and serve as an internal control for the amount of DNA loaded per lane on a Southern blot (Nottenburg and Weissman, 1981; Coleclough *et al.*, 1981). The *Bam*HI site polymorphism described by Sheppard and Gutman (1981b) was not used because in LEW rats J_k and C_k are found on the same *Bam*HI fragment, precluding the use of this internal control. Liver DNA from DA and LEW rats was digested with *Bgl*II, *Cla*I, *Hinc*II, *Hpa*I, *Pst*I, *Sal*I, *Sma*I, *Sst*I or *Xho*I restriction endonucleases, electrophoresed on 0.7% agarose gels and transferred to nitrocellulose paper. The blots were hybridised with either prJ1/2, the J_k probe, or prC_k, the C_k probe. Only *Pst*I revealed a size difference between DA and LEW when probed with prJ1/2. J_k-containing bands appeared at 8.6 kb for DA and 6.0 kb for LEW. For C_k there was a single band at 7.9 kb (Figure 2). Confirmation that the J1/2 probe was detecting only kappa-related sequences came from the observation of identical restriction patterns in DA and LEW-*Igk-1*^{b}; the 8.6-kb band had survived 10 generations of genetic segregation in backcrosses selected by the anti-Igk-1^b allotype reagent.

prJ1/2 does not hybridise to J_k3 or J_k4 (Sheppard and Gutman, 1981a). Because V segments are 5′ to the J segments to which they rearrange, it was critical to demonstrate that J1 and J2 truly map to the 5′ side of J3 and J4. This was confirmed by preparing a probe for J3/4 from the 6.5-kb *Eco*RI clone of Sheppard and Gutman (1981a) and showing that this probe hybridised to an *Eco*RI fragment of the same size (6.5 kb) as that binding prC_k and to a *Pst*I-*Bam*HI co-digested fragment of the same size (3.5 kb) as that hybridising to prJ1/2. No other bands could be seen with either prJ1/2 or prJ3/4.

Quantitation of rearrangement

B cells from a (LEW x DA)F_1 heterozygote were prepared by rosetting to remove W3/13-positive cells (T cells and B blasts). DNA was extracted, cut with *Pst*I, electrophoresed, blotted to nitrocellulose and probed with mixed labelled prJ1/2 and prC_k. Standards were prepared in which B cell DNA was titrated into sperm DNA in known proportions. These standards were compared with DNA from homozygous DA and LEW sperm DNA having a double dose of J_k^b and J_k^a genes, respectively. The J_k and C_k band intensities were estimated by densitometer. In the homozygotes the germ-line J_k bands were $\sim 3-4$ times as intense as the C_k band, making it relatively easy to estimate the loss of the J_k band – even of the heterozygote where each J gene is present in only half the dose of the C_k gene. To allow for the slight variations in the relative intensities of J_k and C_k on each nitrocellulose filter the J_k/C_k ratios were normalised by dividing by the J_k/C_k ratio for F1 sperm. Therefore, the normalised ratio for homozygous germ-line DNA ought to be 2.0; we found 1.5 for DA, 1.6 for LEW-*Igk-1*^{b} and 2.3 for LEW (Table I). For the B cell DNA, substantial rearrangement of both alleles was indicated by the normalised ratios of 0.21 (J_k^b/C_k) and 0.23 (J_k^a/C_k), showing the loss of the J_k bands relative to sperm DNA. Table I also demonstrates that the samples containing mixtures of sperm and B cell DNA had intermediate values for the normalised ratios roughly in agreement with expectation. These standards therefore give

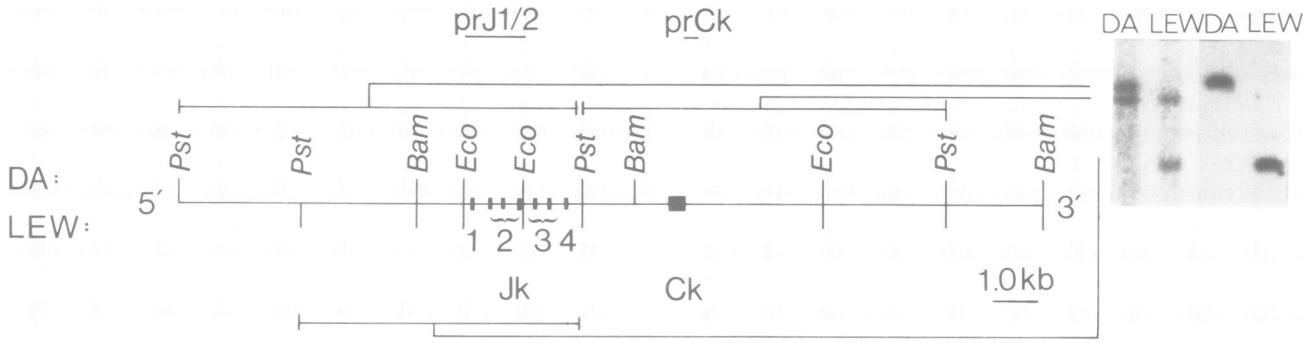

Fig. 2. Southern blots of liver DNA from LEW and LEW-*Igk-1*^{b} cut with *Pst*I and probed with prJ1/2 (right), or mouse p C_k (left) which cross hybridises with both C_k and J_k of the rat. Note the dimorphism of J1/2 fragment lengths. Restriction sites may be derived from these data and Sheppard and Gutman (1981a).

Table I. Values for the ratio of J_k/C_k in germ-line DNA and in B cells. Densitometry values for J_k^a, J_k^b and C_k bands

Source of DNA	Ratio J_k/C_k[1]			
	J_k^b/C_k		J_k^a/C_k	
	Expected	Observed	Expected	Observed
DA sperm	2.0	1.54	0	0
LEW *Igk-1*^{b} sperm	2.0	1.61	0	0
LEW sperm	0	0	2.0	2.32
F_1 sperm:F_1 B cells (50:50)	0.61	0.69	0.62	0.58
F_1 sperm:F_1 B cells (25:75)	0.41	0.53	0.42	0.73
F_1 B cells[2] (no sperm)		0.21		0.23

[1]Because the F_1 sperm J_k/C_k ratios varied a little from one filter to another, normalization was performed for each filter before averaging. Mean sperm J_k^b/C_k = 2.08 ± 0.58; J_k^a/C_k = 1.95 ± 1.04.
[2]B cells prepared by rosetting to remove W3/13$^+$ cells only: no enrichment for allotype.

some idea of the precision of the method of assay in our hands. Our value for the residual germ-line component in total B cells is approximately similar to but rather lower than the figure of 0.35 obtained by Coleclough *et al.* (1981) and the value calculated from data obtained by Joho and Weissman (1980) in the mouse (see Discussion).

Isolation and DNA analysis of Igk-1^a *and Igk-1*^{b} *expressing B cells*

In these experiments, (LEW x DA)F_1 B cells were similarly prepared by rosetting and depletion (experiments 1 and 2, Table II) (Figure 3) or, to give greater purity, by further staining with fluorescein-labeled anti-allotype antibody before sorting on the FACS (experiments 3 and 4, Table II). Purities >92% were found by re-analysis after the sorts. To check whether some non-surface synthesis of the opposite allotype might be occurring (which would have required some rearrangement of the apparently non-expressed chromosome), post-sort samples were metabolically labelled with [^{35}S]-methionine and analyzed by immunoprecipitation with each anti-allotype antibody. Because most immunoglobulins are synthesized by B-lymphoblasts, which were removed by rosetting, only a small though measurable amount of radioactivity was immunoprecipitated. The results confirmed the purity of the sorts and proved the absence of any detectable internal synthesis of the 'wrong' allotype.

The slightly different methods of preparation of the Igk-1^a and Igk-1^b expressors, which might have been expected to have been contaminated by λ-bearers and by a$^+$b$^+$ double-synthesisers as indicated in Table II, proved to cause no systematic observable variation in the normalised J_k/C_k ratios, and the results can be considered together as a group. The four samples of Igk-1b$^+$ cells showed a mean of 0.06 of

Table II. Observed ratio of J_k/C_k on the expressed and non-expressed chromosome. Densitometry values for J_k^a, J_k^b and C_k bands

Expt. No.	Source of DNA	Purity %	Observed ratio[1] J_k/C_k	
			J_k^b/C_k	J_k^a/C_k
1	*Ig-1*$^{b+}$ F_1B cells[2]	85	0.05	0.55
2	*Ig-1*$^{b+}$ F_1B cells[2]	81	0.10	0.45
3	*Ig-1*$^{b+}$ F_1B cells[3]	92	0.04	0.52
4	*Ig-1*$^{b+}$ F_1B cells[4]	92	0.06	0.61
		Mean ± SD =	0.06 ± 0.03	0.53 ± 0.07
4	*Ig-1*$^{a+}$ F_1B cells[4]	96	0.38	0.09

[1]Normalized to the J_k/C_k ratio for the allotype on F_1 sperm.
[2]B cells expressing single allotype prepared by rosetting to remove W3/13$^+$ cells and MAR-80$^+$ cells. W3/13 is anti-T cell and anti-B lymphoblast; MAR-80 is anti-*Igk-1*^{a}*1*. Possible contamination with λ$^+$.
[3]Same procedure as experiments 1 and 2, then sorted by FACS following restaining with anti-*Igk-1*^{b} antibody.
[4]B cells prepared by rosetting to remove W3/13$^+$ cells, then sorted following MAR-80 staining by FACS into negative (containing *Igk-1*$^{b+}$) or positive *(Igk-1*$^{a+}$*)* fractions. Possible contamination with λ$^+$ for *Igk-1*$^{b+}$ and with a$^+$b$^+$ for *Igk-1*$^{a+}$.

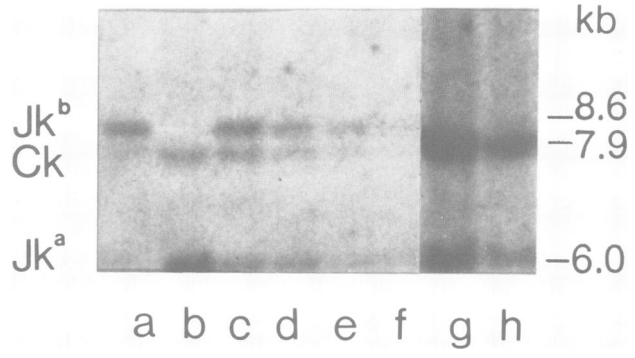

Fig. 3. Autoradiograph (14 days exposure) of Southern blot of sperm and purified B cell DNA after probing with a mixture of prJ1/2 and pr C_k. **a**, DA sperm; **b**, LEW sperm; **c**–**f**, (LEW x DA)F_1 sperm, 10, 5, 2, 5, 1.2 μg; **g,h**, F_1B cell (expressing only *Igk-1*^{a}) samples 1 and 2. Note the disappearance of the band due to the expressed allotype (8.6 kb in **lanes g,h)** and the reduction, but not complete loss, of the non-expressed allotype (6.0 kb).

the germ-line J_k^b/C_k ratio indicating almost complete rearrangement; in the other direction the single sample of Igk-1a$^+$ likewise showed 0.09 of the J_k^a/C_k ratio. The residual germ-line component is most likely to be explained by the impurity of the preparations. For the non-expressed chromosome the values were 0.53 for J_k^a/C_k in the Igk-1b$^+$ cells and 0.38 for J_k^b/C_k in the single Igk-1a$^+$ preparation. These values are far too low to be explained either by contamination by double-stainers or by λ-positive cells, even if both these contaminants had rearranged both k genes (Hieter *et al.*, 1981; Alt *et al.*, 1980). The conclusion is firm then that half or more of the non-expressed kappa genes have rearranged their J_k gene segments.

Discussion

One aim of this study was to place an upper limit on the frequency of B cells capable of synthesising both kappa allotypes, which would be cells in which both chromosomes are rearranged. Our figure of 8% double-stainers (among B cells) is higher than would have been predicted for peripheral lymphocytes, by previous studies in the rabbit (Pernis *et al.*, 1970; Jones *et al.*, 1973), though this is entirely compatible with a previous single-staining observation in the rat (Hunt and Williams, 1974). The difference is probably attributable to the use of the FACS for analysis, which is not subject to the problems of quantitation and fading of fluorescein fluorescence that afflict direct visual inspection. The FACS can also analyse many more cells to improve the statistical significance of the data. The problem of whether double staining should be interpreted as double-synthesis or cytophilic antibody was discussed in the results section and has been treated by others earlier (Jones *et al.*, 1973; Loor and Kelus, 1978), with no satisfactory resolution. Perhaps an allophenic rather than a radiation homozygous mixed chimera would be a better control. Accurate estimates of genuine double synthesisers would provide important information about the relative frequency of productive versus non-productive rearrangements in normal B cell populations (Coleclough, 1983) but for the present purpose determining the maximum percentage suffices.

Our results show that rearrangements to the phenotypically silent kappa chromosome do occur in normal B lymphocytes. The incidence of double expressors is too low to account for the substantial disappearance (>60%) of the germ-line context noted in previous studies of total B cells not sorted for allotype (Joho and Weissman, 1980; Coleclough *et al.*, 1981; Van Ness *et al.*, 1982) and confirmed here. The ability to sort for cells expressing a single allotype has now allowed the direct determination of the degree of rearrangement of the non-expressed chromosome. It was found to be ~50% for Igk-1b$^+$ cells and perhaps even higher (~60%) for the single sample of Igk-1a$^+$ cells, though this difference could be explained partly by the inclusion of some double-expressors in the positively-sorted Igk-1a$^+$ fraction. Therefore rearrangement of only one chromosome cannot be the sole explanation of light chain allelic exclusion as suggested by Joho and Weissman (1980). Table I of Joho and Weissman (1980), referring to total murine kappa-positive cells, gave 41% for the residual germ-line component. When reinforced by two further independent estimates of 30–35% (Coleclough *et al.*, 1981; Coffman and Weissman, 1983) this suggests that some rearrangement of the silent allele did occur though the original estimate was not precise enough to be sure that it differed significantly from the 50% interpreted as unaltered germ-line arrangement of the non-expressed allele. Rearrangements to the phenotypically silent J_k-C_k region appear to occur more often in this study of the rat as compared to the mouse. It is possible that these more frequent rearrangements in the rat are related to a greater target size for the rat (seven J_ks; Burstein *et al.*, 1982; Sheppard and Gutman, 1981a, 1981b, 1982) than the mouse (five J_ks), as suggested by Shechter (Burstein *et al.*, 1982). Many of the rearrangements must be non-productive, giving rise to termination codons or incorrect mRNA splicing as shown for heavy chains in the mouse (Early *et al.*, 1982), and a signal preventing further rearrangement (Lewis *et al.*, 1982) could then operate when a functional rearrangement has occurred. This signal may be 'leaky' enough to allow rather more double-expressors than has previously been assumed. Alternatively a random process could be operating with a fairly high ratio of non-productive to productive rearrangements, terminated before all germ-line context had been lost. The double expressors could either result from further alterations to a non-productively rearranged chromosome, as has been shown in an Abelson-transformed cell line (Lewis *et al.*, 1982), or from two initial productive rearrangements.

Rearrangement of the expressed chromosome was, as expected, almost complete. The small residual germ-line component is probably attributable to contamination by non-B cells and can perhaps be used to give an idea of the overestimate of the germ-line context of the non-expressed chromosome due to that cause. This observation rules out any substantial genetic exchange between the two homologous chromosomes of non-sister chromatids, and confirms for kappa chains the earlier deduction from serological studies of rabbit heavy chain allotypes that integration is *cis* not *trans* (Landucci-Tosi *et al.*, 1970; Pernis *et al.*, 1973). If exchange had occurred between homologous chromatids with retention of the reciprocal fragment, the latter would have contributed J_k of the 'expressed' allotype in a germ-line context. *Intra*chromosomal mechanisms of rearrangement involving either deletion (with or without inversion (Lewis *et al.*, 1982)) or unequal sister chromatid exchange (Van Ness *et al.*, 1982) or recombination between homologous chromosomes before replication at S phase can all be accommodated with the present data.

Overall, the temporal (Coffman and Weissman, 1983) and quantitative (Nottenburg and Weissman, 1981) differences between control of rearrangements of heavy and light chain genes in normal pre-B cells may be ones of degree rather than kind. A potential confounding element in these kinds of analyses has been introduced by the recent findings of *inter*chromosomal rearrangements in tumor cells in which either heavy chain genetic material (Van Ness *et al.*, 1983) or non-immunoglobulin associated c-*myc* DNA (Taub *et al.*, 1982; Adams *et al.*, 1982; Favera *et al.*, 1983; Shen-Ong *et al.*, 1982; Crews *et al.*, 1982; Harris *et al.*, 1982) translocates to immunoglobulin (sometimes heavy, sometimes light) chain gene sequences. The consequence would again be the loss of the $J_{(k\ or\ H)}$ germ-line context from the non-expressed homologous chromosomes. Because it has been proposed that the interchromosomal translocation of c-*myc* sequences is responsible for the malignant behaviour of the cells one might expect that this mechanism only contributes to silent chromosome rearrangements in these tumors. If, however, this can occur in cells as relatively immature as B cells rather than just in end-stage B lymphomas and plasmacytomas, the trans-

location partner of the non-expressed allele in allelically excluded rat B cells could be identified by analysis of genomic libraries from these cells.

Materials and methods

DA, LEW and LEW-*Igk-1*^{b} (N10) highly inbred rats and their F_1 hybrids were from our own colony at the Stanford University Medical Center or from the sublines established by Dr. G.Gutman, University of California at Irvine. Both males and females over 3 months old were used. Radiation chimeras were prepared as antibody staining controls (Figure 1) by i.v. injection of 7 x 10^6 LEW bone marrow cells into LEW-*Igk-1*^{b} hosts given 600 rad whole-body X-irradiation 6 h previously. They were used >3 months later.

Antibodies

Anti-Igk-1^a was monoclonal IgG made by immunoadsorption on LEW IgG-Sepharose from MAR-80 culture supernatant (Lanier *et al.*, 1982) (gift of G.Gutman). It was biotinylated by mixing the protein at 1 mg/ml in bicarbonate buffer pH 8.4 with biotin succinimide ester (1 mg/ml freshly dissolved in dimethylsulfoxide) at a ratio of 1:0.18 (v/v) for 4 h at 20°C, then removing hydrolysed unreacted ester by chromatography on Sephadex G-25. Anti-Igk-1^b was the direct fluorescein-conjugated $(Fab')_2$ polyclonal alloanti IgG, purified by immunoadsorption and previously described (Hunt and Fowler, 1981). W3/13 IgG (monoclonal antibody to rat T cells) (Mason *et al.*, 1980) was the generous gift of Dr. W.R.A.Brown, Dunn School of Pathology, Oxford, UK.

$(Fab')_2$ rabbit anti-mouse Ig and its fluorescein conjugate were kind gifts from Dr. A.F.Williams, Dunn School of Pathology, Oxford. Texas Red-avidin conjugate was a generous donation from Dr. G.Kraal, Pathology Department, Stanford.

Cell preparation

Thoracic duct lymph (Ford, 1978) was collected on ice for periods up to 20 h: cells were harvested after 1–4 days of drainage. Grossly bloody samples were discarded. In a few experiments, superficial and deep cervical and mesenteric lymph nodes were taken and dissociated by teasing with watchmaker's forceps, then centrifuged over Metrizoate-Ficoll (density 1.078 gm/ml) to remove dead cells. All subsequent manipulations were performed on ice in the presence of 10 mM NaN_3 in Dulbecco's A + B solution containing 2% newborn calf serum. Suspensions were washed three times, then stained for 60 min at just saturating antibody concentrations.

To prepare B cells, lymphocytes were labelled with W3/13 IgG, washed and rosetted with sheep erythrocytes coated by the chromic chloride procedure with rabbit-anti-mouse Ig (Hunt, 1978). Rosettes were removed by centrifugation over Metrizoate-Ficoll. The floating cell layer was washed and either used directly or restained for sorting. Single-colour fluorescence analysis and sorting was done on a FACS III using 488 nm excitation; two-colour fluorescence was measured on a modified FACS II with sequential 488 (argon laser) and 582 nm (dye laser) excitation (Parks *et al.*, 1983). Post-sort purities were checked by immediate re-analysis and in the case of the dull fractions by restaining with the opposite anti-allotype.

DNA preparation, digestion and hybridisation

Cells were lysed in 1% w/v lauroyl sarcosine at 50°C for 2 h with ~0.1 mg/ml Proteinase K (Beckman). After equilibration with ethidium bromide to a final 1% w/v the lysate was centrifuged through CsCl of density 1.20 g/ml to a shelf of saturated CsCl (4 h, 35 000 r.p.m. SW50.1 rotor). After butanol extraction of ethidium and dialysis the DNA was stored in 10 mM Tris pH 7.5, 1 mM EDTA at 4°C.

Restriction enzymes were purchased from New England Biolabs and used to digest 5–10 μg DNA under the conditions recommended by the supplier. Flat-bed agarose gel (0.7%) electrophoresis, depurination with acid, denaturation with alkali and Southern blotting to nitrocellulose (Schleicher and Schuell, 0.45 μ) were performed according to Davis *et al.* (1980). After baking at 80°C for 2 h, filters were wetted in hybridisation solution [50% v/v formamide (MCB), 0.5% (w/v) SDS, 0.1 mg/ml] with denatured salmon sperm DNA (a generous gift of H.Gershenfeld), 0.9 M NaCl, 0.05 M sodium phosphate pH 7.0, 5 mM EDTA ('5 x SSPE'). ^{32}P-labelled plasmid DNA containing the appropriate probes (nick-translated to >2 x 10^8 c.p.m./μg DNA (estimated by absorption at 260 nm) was added to a final concentration of ~5–10 ng/ml in a heat-sealed plastic bag and hybridised for 16–40 h at 42°C. Filters were washed at 50°C in 1 x SSPE for 1–3 h and exposed at −70°C to Kodak XAR x-ray film with a DuPont lightning-Plus intensifying screen.

Probes (Figure 2)

prJ1/2 was a subclone into pBR325 of a 1.3-kb *Eco*RI piece of the 13-kb LEW genomic cloned fragment containing C_k and J_k (Sheppard and Gutman, 1981b). It contains the J1, J2a, J2b and J2 coding sequences plus ~150 bp 5′ to J1 (Sheppard and Gutman, 1981a).

prC_k was a subclone into a *Hae*III site of pBR322 of a 0.9-kb triple-repeat of 300-bp *Sau*3A genomic fragment of LOU origin (Sheppard and Gutman, 1981a). It contains the 3′ half of the C_k coding sequence plus most of its 3′-untranslated sequence.

Densitometry

The region of interest of each track on the X-ray film was scanned with a Helena Laboratories quick-scan densitometer (Model 1038) (generously loaned through Dr. R.Coffman, DNAX, Palo Alto) with the gain set such that the most intense band gave a full-scale deflection. The slit was set so as to scan about half the width of each track and each track was usually scanned twice, on each half, except where obvious background spots interfered. The measurements for each half were pooled. Each author independently determined the area under each peak on photocopy duplicates of the densitometer trace. The ratios of the areas under each J_k band to the C_k band were calculated. The data given in Table I are the mean ratios of the duplicates performed for each DNA sample.

The exposure time of the autoradiographs was judged sufficient when the signal strength of the weakest band doubled when the exposure time was doubled (allowing for ^{32}P decay).

The whole procedure, digestion, blotting, hybridising and autoradiographing, was performed at least twice for each DNA sample.

Nomenclature

In this paper the rat kappa allotype nomenclature agreed in 1982 [Gutman *et al.* (1983) *Transpl. Proc.*, **15**, 1685] is followed. The notation of the *a* and *b* alleles is the inverse of the system we have followed in earlier publications.

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