Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 1438–1442, March 1986
Immunology

Frequent λ light chain gene rearrangement and expression in a Ly-1 B lymphoma with a productive κ chain allele

(lipopolysaccharide-induced B-cell differentiation/cytofluorometry/RNA/DNA/isotype exclusion)

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Contributed by Leonard A. Herzenberg, September 30, 1985

ABSTRACT We describe here a murine Ly-1-bearing pre-B-cell tumor that, when induced for κ light chain expression with bacterial lipopolysaccharide, also gives rise spontaneously to a few percent of cells expressing surface λ light chains. These λ-positive cells have undergone DNA rearrangements involving either V_{λ_1} or V_{λ_2} genes. Nearly all clones of λ-bearing cells express μ and λ on their surface (but not κ). However, all these λ-positive clones continue to transcribe κ mRNA and synthesize internal κ chains. Further, surface λ-positive clones show J_H rearrangements on one or both heavy chain chromosomes.

The molecular events of DNA rearrangement at the immunoglobulin heavy (H) and light (L) chain loci have been extensively investigated in various B-cell lines and tumors (1). During B-cell differentiation, first the immunoglobulin heavy chain locus and then the light chain locus rearranges to associate one of a number of specificity-determining variable (V) region sequences with a constant (C) region sequence. B cells express either κ or λ light chains, a phenomenon generally believed due to productive rearrangement of a κ allele blocking further light chain gene rearrangement. This model accounts for the common findings that a single B cell expresses one V_H gene and either a κ or a λ light chain. However, recent data suggest that B cells can be divided into subpopulations (2, 3) that may constitute distinct developmental lineages (4–6). Since different DNA rearrangements might characterize each lineage, attempts to establish a single molecular mechanism of DNA rearrangements during B-cell differentiation could lead to contradictions.

Several years ago Lanier *et al.* (7) demonstrated that the murine pan-T antigen Ly-1 was also expressed on certain B-cell tumors. More recently we have shown that Ly-1 is expressed on a functionally distinct subset of normal B cells, "Ly-1 B" (3, 6). The many differences observed between Ly-1 B and the Ly-1-negative B cells (including differences in surface antigen expression, appearance during development, tissue distribution, and responsiveness to antigens) led us to suggest that Ly-1 B constitutes a distinct lineage of B cells (3, 4, 6). Indeed, cell transfer assays have recently demonstrated that the precursors for B cells lacking Ly-1 are distinct from those for Ly-1 B (6). Investigations of homogenous populations of Ly-1-positive B cells might reveal differences between these and Ly-1 negative B cells at the molecular level.

We have begun such investigations with NFS-5, a Ly-1-positive pre-B tumor (8) inducible for κ light chain expression by treatment with the B-cell mitogen lipopolysaccharide (LPS) (9). In contrast with the model for DNA immunoglobulin gene rearrangements described above, we have found frequent rearrangement and expression of λ genes in κ-positive clones of this line. Remarkably, these λ-expressing clones are still capable of surface κ expression and, moreover, have new productive rearrangements of the heavy chain joining region (J_H) on previously expressed heavy chain chromosomes.

MATERIALS AND METHODS

Slot Blot RNA Hybridization. Total cellular RNA was prepared as described in ref. 10. For slot blot hybridization (11), 40 μg of RNA was denatured for 15 min at 60°C in a total volume of 100 μl containing 30 μl of 20× SSPE (1× SSPE = 0.15 M NaCl/0.01 M sodium phosphate/0.001 M EDTA, pH 7.4) and 20 μl of formaldehyde (37% wt/vol, Fisher). The 100 μl was then diluted by addition of 300 μl of 15× SSPE. Four-fold serial dilutions were made, and 100 μl of each dilution was applied to nitrocellulose through a slot blot apparatus. Each well was rinsed once with 100 μl of 15× SSPE. The filters were baked, prehybridized, hybridized, and washed as described below for Southern blots. Probes used were C_κ, the 2.6-kilobase (kb) *Bam*HI/*Hin*dIII fragment containing the C_κ exon (12), and V_{λ_1}, a cDNA probe that detects V_{λ_1} and V_{λ_2} (13). Densitometry was performed on slots containing 10 and 2.5 μg of RNA. Tracings from duplicate experiments were weighed in triplicate, and values were normalized to background hybridization of each probe to kidney RNA.

Cell Lines and Culture Conditions. Cells were cultured in RPMI 1640 medium supplemented with antibiotics (penicillin, streptomycin), 2-mercaptoethanol (50 μM), and 10% fetal calf serum and were maintained in a humidified, CO_2-gassed (5%) 37°C incubator. Cells were either continuously maintained in medium containing LPS at 10 μg/ml or removed from LPS for 10 days and then passed back into LPS 3 days prior to analysis (to examine reinduction of light chain).

Fluorescence-Activated Cell Sorter (FACS) Analysis and Sorting. Cells were stained with fluorescein-labeled goat anti-λ together with biotin-labeled monoclonal anti-κ (antibody 187.1, ref. 14)/Texas red avidin and analyzed on a dual laser FACS as described previously (15). Cells were cloned by using a device capable of depositing single cells of a desired phenotype into 96-well culture plates. All immunofluorescence data are presented on a logarithmic scale of approximately four powers of 10. Dead cells were eliminated by propidium iodide gating. Contour plots presenting relative frequencies of cells at particular ratios of red and green stain were generated from list mode data collected on

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Abbreviations: V, variable; C, constant; J, joining; D, diversity; FACS, fluorescence-activated cell sorter; kb, kilobase(s); LPS, lipopolysaccharide.

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a VAX-11/780 computer using software designed in this laboratory by W. Moore.

Southern Blots of Genomic DNA. Genomic DNA (10 μg) was digested for 6–12 hr with a 5-fold unit excess of enzyme in buffer conditions recommended by the supplier (New England Biolabs). Agarose gel (0.7%) electrophoreses were run in standard Tris/borate buffer at 20 V for 24 hr. After blotting, filters were baked at 80°C for 2 hr and prehybridized overnight at 65°C in 6× SSPE/0.1% $NaDodSO_4$/2× Denhardt's solution containing denatured salmon sperm DNA at 100 μg/ml. Hybridization was carried out for 18 hr in the same solution, plus 10^6 dpm/ml of each probe, nick-translated to 1–3 × 10^8 dpm/μg (radionucleotides from New England Nuclear; nick-translation kit from Bethesda Research Laboratories). Two 20-min washes in 2× SSPE/0.1% $NaDodSO_4$ followed by two 20-min washes in 0.2× SSPE/0.1% $NaDodSO_4$ at 65°C sufficed to eliminate nonspecific backgrounds. Exposure times were typically 1–3 days with preflashed Kodak XAR-5 film and DuPont Cronex Lightning Fast intensifying screens.

$NaDodSO_4$/PAGE Analysis of Surface-Labeled Cell Immunoprecipitates. Samples consisting of 1–2 × 10^7 cells were labeled with 1 mCi (1 Ci = 37 GBq) of ^{125}I by lactoperoxidase-catalyzed iodination (16); cells were then lysed with Nonidet P-40 extract buffer for 30 min on ice, the extract was centrifuged, and the supernatant was harvested for immunoprecipitation. Fixed *Staphylococcus aureus* strain A cells precoated with anti-rat κ (MAR 18.5, ref. 17) were coated with second-step precipitating antibody (anti-μ 331.12, ref. 18, or anti-λ JC5, a gift of J. Kearney, University of Alabama) and then added to cleared extracts; after washing, staph pellets were extracted with $NaDodSO_4$ sample buffer (containing dithioerythritol as a reducing agent) and analyzed by $NaDodSO_4$ electrophoresis in 10% acrylamide. Labeled proteins were detected by autoradiography after a 4-day exposure using Kodak XAR-5 film with intensifying screen at −70°C.

Two-Dimensional Electrophoresis of Metabolically Labeled Cell Immunoprecipitates. Samples consisting of 1–2 × 10^7 cells in 5 ml of methionine-free RPMI 1640 medium with 10% fetal calf serum were biosynthetically labeled with 1 mCi of [^{35}S]methionine (Amersham, 23.5 mCi/ml) for 4 hr. Precipitations were carried out as described above, using either rat anti-mouse κ (187.1) or anti-mouse μ (331.12). After washing, the staph pellets were extracted with first-dimension buffer (nonequilibrium pH gradient) containing dithiothreitol as a reducing agent; electrophoresis in the first and second dimensions followed published procedures (19). Labeled proteins were detected after 5 days of salicylate-enhanced autoradiography using Kodak XAR-5 film together with intensifying screens at −70°C.

RESULTS

The NFS-5 κ-negative parental clone, which expresses normal-size μ heavy chain on its surface (unpublished data), is termed NFS-5.3 and its κ-positive clonal progeny, NFS-5.4K. One of many similar λ-positive clones derived from NFS-5.4K was selected for further analysis (NFS-5.4L), and a unique κ/λ double-expressing clone (1 found among 30 λ clones analyzed), termed NFS-5.4KL, was also examined. All λ-expressing clones require the continued presence of LPS for expression of light chain, as was found with NFS-5.4K. That is, λ-expressing clones lose light chain expression

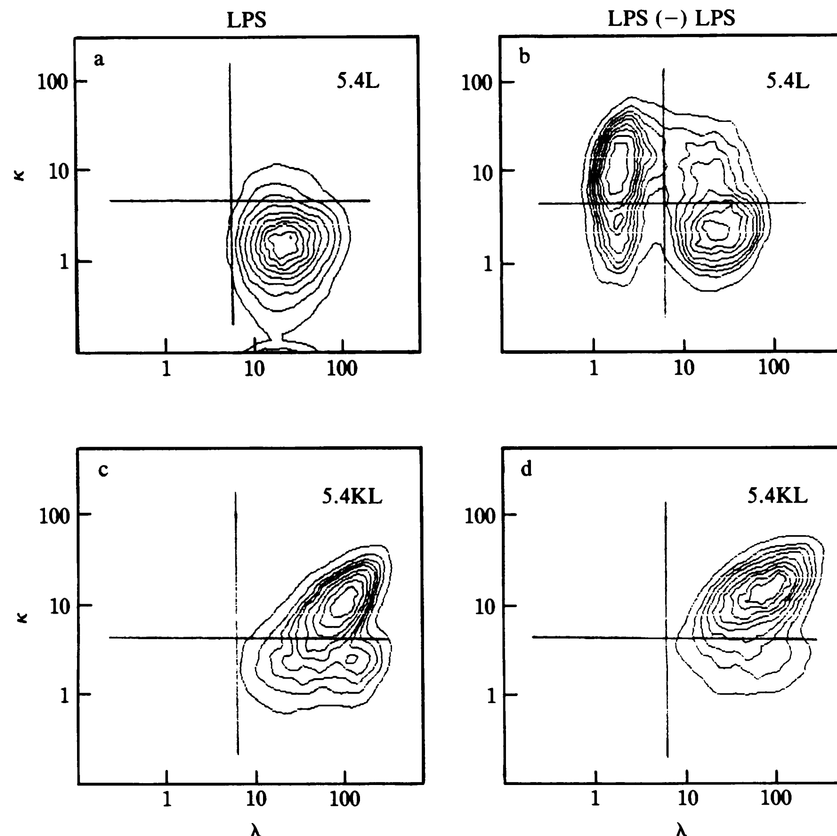

FIG. 1. Two-color FACS analysis of expression and reexpression of light chains in λ-positive clones after withdrawal of LPS and restimulation with it. NFS-5.4L is a representative λ-positive κ-negative clone; NFS-5.4KL is the unique κ/λ double-expressing clone. (*Left*) The two clones prior to removal from LPS medium. (*Right*) The two clones after reinduction of light chain expression by return to LPS medium. Perpendicular lines drawn on the plots show cutoff between positive and negative. Numbers on the axes are relative fluorescence intensity; contour intervals are drawn such that 10% of cells fall between adjacent contours.

after culture for 1 week in medium lacking LPS. After reinduction of light chain with LPS, typically about 40% of the cells expressed κ (with no λ) and about 50% of the cells expressed λ (with no κ). Therefore, at least one κ allele is still expressible in the lines that contain productively rearranged λ genes. Furthermore, a small population (about 5%) simultaneously expresses both light chains. When NFS-5.4KL was treated similarly, both κ and λ rapidly reappeared on all cells (Fig. 1).

Analysis of genomic DNA by Southern blotting (20) shows the state of the immunoglobulin light chain gene rearrangements in the NFS-5-derived cell lines (Fig. 2). Both alleles of κ are rearranged in the parental tumor (data not shown). There are no changes in the restriction digest patterns revealed with a J_κ probe in any of the clones we have generated (Fig. 2*a*). This is true even after removal of LPS and reinduction with it (not shown). In contrast, the λ locus is seen to rearrange only in the two λ-expressing lines analyzed (Fig. 2*b*). The restriction fragment sizes seen with a V_λ probe indicate that NFS-5.4L is rearranged to λ_2, while NFS-5.4KL is rearranged to λ_1 (23). Thus, in the two λ clones analyzed, two different λ rearrangements have occurred.

Unexpectedly, both productive and nonproductive heavy chain alleles undergo continued rearrangement. In the original tumor, rearrangements of 6.3 kb and 5.7 kb are detected upon digestion with *Eco*RI and probing with J_H (24). Of two 5.3 clones analyzed, one (Fig. 2*c*, lane 2) retains the 6.3-kb allele but not the 5.7-kb allele; the other (not shown) retains both. This argues that the 6.3-kb fragment represents the expressed allele. NFS-5.4K has the 6.3-kb allele, and another smaller rearranged J_H fragment (Fig. 2*c*, lane 3). Upon switching of NFS-5.4K from κ to λ surface expression, both J_H alleles can show further rearrangement. NFS-5.4L maintains the expressed 6.3-kb allele seen in clones NFS-5.3 and NFS-5.4K (confirmed by using two other restriction enzymes; not shown) and in addition shares a new 6.6-kb allele with NFS-5.4KL (Fig. 2*c*, lanes 4 and 5). Presumably, this reflects further rearrangement on the nonexpressed chromosome. In addition to the new nonexpressed allele, NFS-5.4KL also has a unique second J_H allele (Fig. 2*c*, lane 5) instead of the 6.3-kb allele. Further restriction analysis (not shown) confirms that this new, smaller allele represents a truly novel rearrangement, and not simply a deletion within the 6.3-kb allele (see *Discussion*). As with the κ locus, no changes in J_H rearrangement are seen after further cycles of culture with and without LPS (not shown).

Immunoprecipitation analysis of ^{125}I-surface-labeled (16) NFS-5 clones (Fig. 3) demonstrates that the λ chains from two λ clones migrate differently, as would be expected for two distinct λ subtypes. This is in agreement with DNA rearrangements observed in these cell lines and is further

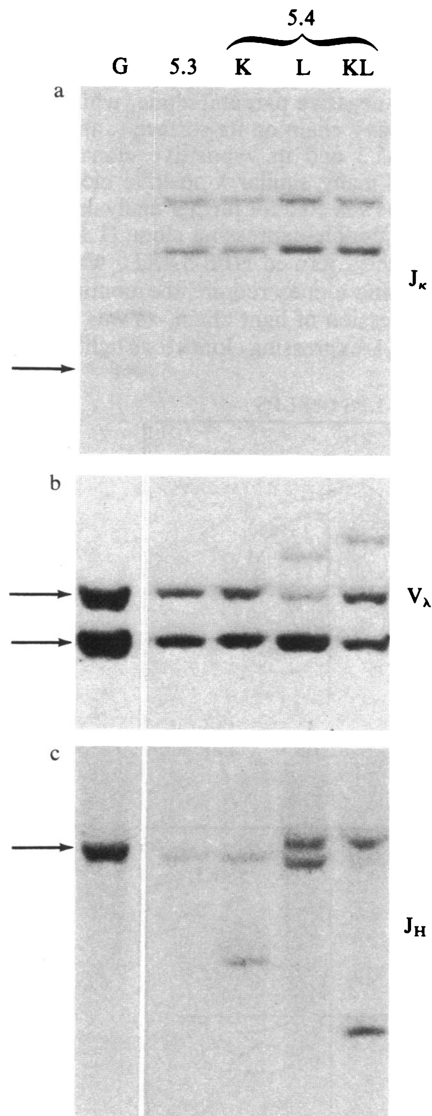

FIG. 2. Southern blot analysis of immunoglobulin gene rearrangements in NFS-5-derived clones. Total genomic DNA from NFS-5-derived cell lines and BALB/c liver (lanes G) was digested and probed as follows: (*a*) Digested with *Hin*dIII, probed with a 1.0-kilobase (kb) *Xba* I/*Hin*dIII J_κ probe (21). (*b*) Digested with *Eco*RI, probed with a V_λ cDNA probe that detects both V_{λ_1} and V_{λ_2} (13). (*c*) Digested with *Eco*RI, probed with a 0.6-kb *Xba* I/*Eco*RI J_H probe (22). Cell lines are listed across the top; germ-line bands are denoted by arrows.

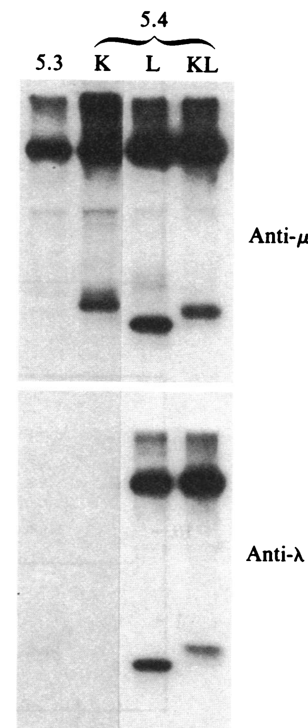

FIG. 3. NaDodSO$_4$/PAGE analysis of immunoprecipitates of surface-radioiodinated NFS-5 clones. Two different λ light chains are expressed on cloned 5.4L and 5.4KL (lanes 3 and 4), and these are specifically precipitated with monoclonal anti-λ antibody (*Lower*). Note that κ and λ light chains precipitated from 5.4KL by anti-μ (*Upper*, lane 4) are not resolved by this gel.

Table 1. Expression of κ and λ RNAs in NFS-5-derived cell lines

Cell line*	Relative amount† of RNA hybridizing	
	C_κ	V_λ
NFS-5.3	1	0.8
NFS-5.4K	5	0.7
NFS-5.4KL	4.8	3.9
NFS-5.4L	4.6	10.9

*Phenotypes of these lines are described in the text.
†For each of the probes, densitometry values are normalized to kidney RNA.

confirmed by two-dimensional gel analysis of biosynthetically labeled light chains (see below).

RNA slot blot hybridization (11) presented in Table 1 shows that the level of κ hybridizable RNA is similar in all light chain-expressing clones analyzed, including one clone that expresses only λ on its surface. To determine whether κ protein is produced intracellularly in all these clones, we carried out two-dimensional gel analysis of κ immunoprecipitates of total cell extracts after biosynthetic labeling with [^{35}S]methionine (19). The results (Fig. 4) show that NFS-5.4K and NFS-5.4L express the same κ light chain, in roughly equivalent amounts. Significantly, anti-κ immunoprecipitation of NFS-5.4L shows that in this line (in contrast with NFS-5.4K and NFS-5.4KL), no internal κ light chain is associated with μ heavy chain. It appears that some κ light chain in NFS-5.4 KL is complexed to a protein that is more basic than μ, but with a comparable molecular weight, whereas all of the κ in NFS-5.4L is so associated. This may be the so-called "heavy chain binding protein" (BiP) previously described by Wabl (22, 25); such a protein (or another similar) might be expected to associate with proteins possessing an "immunoglobulin-fold" tertiary structure (such as light chain).

DISCUSSION

Rearrangement and expression of λ loci in clones still containing a normal, functional, κ gene, we believe, is unprecedented. The λ myeloma lines MOPC104E and HOPC-1 do contain rearranged κ loci and transcribe normal size κ message (26), but these cell lines produce only truncated κ chains intracellularly. There are other instances of κ-secreting myelomas that contain a second κ light chain restricted to the cytoplasm (27, 28). In each of these cases, unusual amino acid residues are found at the V_κ–J_κ joint, rendering these light chains unable to combine with the expressed heavy chain to make tetrameric immunoglobulin. Storb and colleagues (29, 30), in an examination of hybridomas derived from mice containing a microinjected cloned κ gene, concluded that the presence of a functional κ gene suppressed further light chain rearrangements in these hybridomas. Exceptions occurred if the microinjected κ gene was not transcribed or if the κ chain was not associated with heavy chain.

Our results are consistent with a model for light chain rearrangement in which further rearrangement is blocked by formation of intact heavy chain–light chain complexes, if we assume that λ rearrangement is not blocked in the fraction of cells in the κ-positive line that express little or no surface κ (typically 2–5%). However, it is clear that a model of isotype exclusion must address more than simple control of rearrangement since NFS-5.4L (which expresses λ and μ, but not κ, on the surface) contains an internal κ chain that can be reexpressed on the surface after LPS cycling.

The transition from κ to λ expression in Ly-1 B lines may explain the curious observation that many of these lines bear λ light chains. Examples include the BCL_1 lymphoma (31), all lines established by Braun (32), and several "normal B" lines established by Davidson and Morse (33). This transition also may explain the higher proportion of λ expression among normal Ly-1 B cells as compared with the Ly-1-negative B-cell population (35). It may be that short-lived B cells rarely rearrange λ after expressing κ, but either die or are expanded (and switch heavy chain isotype) on exposure to antigen. On the other hand, Ly-1 B cells continue to divide and so can make this transition, increasing to 16–20% λ-expressing cells compared to 4% in Ly-1-negative B cells.

Our finding of a novel rearrangement on a previously expressed heavy chain chromosome suggests the possibility that a new V_H gene is being utilized in a cell line that already contained a productive V_H–D–J_H allele (D, diversity). Fur-

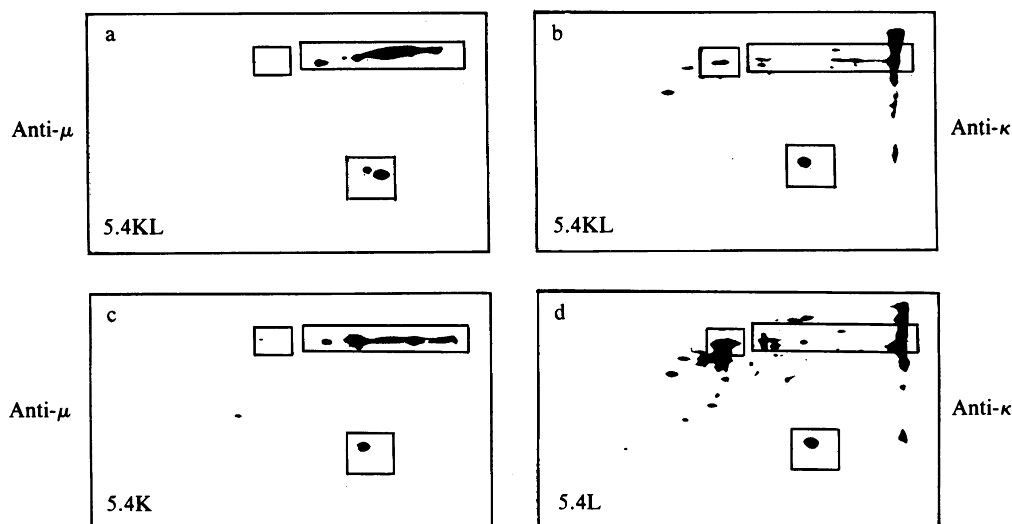

FIG. 4. Two-dimensional gel analysis of immunoprecipitates of biosynthetically labeled NFS-5 clones (nonequilibrium pH gradient electrophoresis, pH 9 to 4; $NaDodSO_4$/10% polyacrylamide gel electrophoresis). NFS-5.4KL has both κ and λ associated with μ (a and b); κ present internally in NFS-5.4L is not associated with μ, but probably with "heavy chain binding protein" (compare c and d). The upper left box marks the region of this putative heavy chain binding protein; the upper right box marks the region for μ heavy chain; the lower right box marks the region for κ light chain.

ther investigation using V_H family-specific probes confirms this startling hypothesis, in that the 6.3-kb allele in NFS-5.3, -5.4K, and -5.4L transcribes a member of the Q52 family, whereas the allele unique to NFS-5.4KL transcribes a member of the 7183 family (R. Kleinfield and M. Weigert, personal communication). This result is difficult to reconcile with current proposals for the mechanism of V_H–D–J_H joining (34). It is intriguing to speculate that the rules governing immunoglobulin rearrangement differ in the Ly-1 B lineage.

Whether the differences we observe in the NFS-5 lines reflect different rules governing molecular events in Ly-1 B differentiation remains to be determined. The major unresolved question posed by these data is how surface isotypic exclusion (only λ on the surface) can be maintained even though κ message and cytoplasmic protein are found in amounts similar to those in surface κ-bearing cells. The κ chain from cells with surface expression appears identical by two-dimensional gel analysis to the κ chain from cells where it is restricted to the cytoplasm. Thus, it appears that in some way assembly of λ with μ is capable of absolutely blocking any assembly of κ with μ. The detection of a large population of κ-only cells in the LPS light chain-reinduced cell line suggests that this process works both ways so that κ association with μ absolutely blocks λ association. Possibly, comparison of clones in which isotypic exclusion occurs (the major type) with the single clone in which both light chains are expressed on the surface will shed light on this puzzle.

We are indebted to Drs. W. Davidson and H. C. Morse III for providing the NFS-5 tumor, Dr. A. Radbruch and Prof. K. Rajewsky for the goat anti-λ antibody, and Dr. H. Nakauchi for probes. We also thank Mr. Tom Nozaki for FACS hardware and Mr. Wayne Moore for software that made these analyses possible. This work was supported in part by Grants GM-17367, HD-01287, and CA-04681 from the National Institutes of Health. R.R.H. was supported by American Cancer Society Senior Fellowship S-12-84. J.L.D. is supported by National Institutes of Health Predoctoral Training Grant 2-T32 GM-07790.

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