FREQUENCY OF EXPRESSED IMMUNOGLOBULIN LIGHT CHAIN GENES IN LIPOPOLYSACCHARIDE-STIMULATED BALB/c SPLEEN CELLS

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The variable (V)[1] regions of the immunoglobulin heavy (H) chain genes are encoded in three groups of linked DNA segments: the variable region (V_H, coding for amino acids 1–101), the diversity region (D_H, coding for the third hypervariable region, amino acids 102–106), and the joining region (J_H, coding for amino acid residues 107–123) (1, 2). Rearrangement of these elements yields a contiguous VDJ DNA segment that leads to a functional H chain gene. The number of germline V region genes was estimated to be ~160 using Southern blotting techniques (3). There are 12 D region germline segments (4) that are believed to be the total D segment repertoire of the mouse. Together with the four J_H regions, the mouse could generate a germline repertoire of 160 × 12 × 4 or ~8,000 V_H genes, assuming random combination of these elements.

For the L chains where no D segments exist, the V_L gene pool was estimated to be ~90 (5). Together with the four functional J_L regions, ~360 rearranged V_L are possible.

Repertoire estimates at the gene level have some intrinsic limitations: it is unknown whether all V (or D) regions are functional and it is not clear whether all V_H-D_H-J_H or V_L-J_L combinations are used with equal frequency. These calculations ignore the variation introduced by the joining process and the somatic mutation of the rearranged genes (1, 2, 6). Therefore, only an analysis of the expressed repertoire will give a more realistic view of the major factors contributing to antibody diversification.

In this paper we describe a way to analyze expressed H or L chains separately, thereby avoiding the enormous heterogeneity created by their combination in antibody molecules. This is achieved by fusing H chain– or L chain–donating hybridoma lines with lipopolysaccharide (LPS)-stimulated spleen cells and screening the resulting hybrids for the restoration of the original H-L antibody specificity.

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[1] *Abbreviations used in this paper:* D segment, diversity segment; 2-D, two-dimensional; HAT, medium supplemented with hypoxanthine, aminopterin, and thymidine; H, L chains, heavy and light chains; IEF, isoelectric focusing; J segment, joining segment; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SRBC, sheep erythrocyte; TNP, trinitrophenyl; V region, variable region.

Materials and Methods

Mice, Cell Lines, and Fusion. Female BALB/c mice, 6–8 wk old, were obtained from the Institut für Biologisch Medizinische Forschung A. G., Füllinsdorf, Switzerland. Spleens were removed and cells were grown in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY) supplemented with penicillin and streptomycin at 100 U/ml each, 10% heat-inactivated fetal bovine serum, 50 μM mercaptoethanol, and 50 μg/ml of LPS (Sigma Chemical Co., St. Louis, MO). After 4 d, cells from one spleen were divided into two parts (4×10^7 cells each) and fused to 2×10^7 hybridoma cells, expressing either the specific H chain together with a nonspecific L chain (HK lines) or expressing the specific L chain (L lines). The lines Sp1, 2, 6, and 7/HLK and their corresponding Sp/HK and Sp/L sublines are described (7). Their specificity and chain combination are summarized in Table II and in Results.

Fusion was performed using 0.7 ml of a 50% (vol/vol) polyethylene glycol, mol wt 4,000 (Merck & Co., Inc., Rahway, NJ), in phosphate-buffered saline (PBS) containing 2 g/l glucose. Cells were divided into two 96-well Costar plates with 0.1 ml culture medium containing 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (HAT). Specific hybrids were cloned on soft agar or by limiting dilution twice or more according to the stability of the hybrids. Anti–sheep erythrocyte (anti-SRBC) or antitrinitrophenyl (anti-TNP) activity was monitored using an anti-Ig–enhanced SRBC agglutination test (8).

The fusion between the unmarked wild-type J558 and the Sp7/HK-Ag^r line was performed as described (9). 2×10^6 J558 cells in 10 ml PBS containing 0.04, 0.16, 0.64, 2.5, and 10 mM iodoacetamide were incubated for 20 min on ice. Each batch was washed twice and fused to 4×10^5 Sp7/HK-Ag^r cells. The J558 cells were effectively killed only when the concentration of iodoacetamide was between 2.5 and 10 mM. Hybrids were selected in HAT medium from these fusions only.

Isoelectric Focusing (IEF) and Two-dimensional (2-D) Gel Electrophoresis. For the 2-D gel analysis, 1 ml of culture supernatant was purified with a rabbit anti-mouse κ antiserum covalently coupled to Sepharose 4B beads. The bound material was eluted from 20-μl beads with 40 μl of solubilizing buffer that consisted of 9 M urea, 2% Nonidet P-40 (British Drug House, Ltd., Poole, England), 1% mercaptoethanol, and 2% ampholines, pH 9-11 (LKB Produkter, Stockholm, Sweden), adjusted to pH 9.5. After spinning down the beads, 20 μl of the supernatant were run on the IEF gels. Details of the particular 2-D system used are published (10–12). Gels were stained by the silver stain method (13). Internally [^{14}C] leucine–labeled culture supernatants (14) were used in some 2-D gels (45-3, 63-34, 1-2 [Fig. 2]) and for the 1-D IEF gels. The dried gels were autoradiographed on Kodak X-Omat film for 6 d.

DNA Analysis. DNA was extracted from ~10^8 cloned hybridoma cells as described (15) except that RNase treatment was omitted. 15 μg of restricted DNA was electrophoresed in 1% agarose gels and blotted onto nitrocellulose according to Southern (16). The mouse κ-specific DNA probe used to reveal V_κ rearrangements is an XbaI/HindIII fragment located 3′ to the mouse J_κ region and was provided by Dr. W. Roeder of this institute.

Results

Four BALB/c-derived hybridoma lines, Sp6/HLK, Sp2/HLK, Sp1/HLK, and Sp7/HLK were used (7). K denotes the kappa light chain contributed by the fusion partner X63-Ag8 or NSI-Ag4/1. The letters H and L denote the heavy and light chains contributed by the B lymphocyte. The HL combination has anti-TNP specificity in Sp6 (IgM, κ) and Sp7 (IgM, λ_1) and confers anti-SRBC specificity in Sp2 (IgG2b, κ) and Sp1 (IgM, κ). From all lines, subclones have been established of the HK and L or LK type; that is, they have lost the specific L or H chain (7). Neither the HK nor the L or the LK combinations retained antibody specificity. Azaguanine-resistant subclones that are HAT sensitive were

selected. These lines were fused either to unstimulated or LPS-stimulated BALB/c spleen cells.

Fusions with BALB/c Spleen Cells. To allow better evaluation of the data, we show the detailed results from the fusions performed with the Sp6/HK line (Table I). The actual number of hybrids was calculated using the Poisson distribution. In those cases where growth was observed in all wells the number of hybrids obtained could not be evaluated. Nevertheless, we give an approximate minimum number of hybrids, assuming one well without hybridoma growth.

In most experiments we were able to restore the original activity. The frequency varied considerably from 1 in 13 to 1 in 330 (Table I, experiment 3 vs. 6). The lower frequencies were obtained preferentially when multiple hybrids grew in the wells and may reflect clonal competition disfavoring the growth and detection of antigen-positive hybridomas. Apparently, similar frequencies of antigen-positive hybridomas were obtained whether or not the spleen cells were LPS stimulated; therefore, we pooled these data.

Table II summarizes all our fusion results, giving the approximate number of actual hybrids. Also included are similar data obtained with the L or LK lines or, in one experiment, the Sp2/0-Ag14 line, which does not contribute any Ig chain. In most cases single spleens were split and fused to the HK and L or LK lines of the same parental origin. Interestingly, the restoration of the H chain–donating hybrids with spleen cell–derived L chains was at least 15-fold (Sp7) to 110-fold (Sp1) more frequent than the restoration of the original specificities of L chain–donating lines with spleen cell–derived H chains. We found no evidence that the restoration was increased over background values using the L or LK lines (e.g., compare Sp2/L and Sp6 or Sp7/LK for anti-TNP restoration, Table II). Pooling the data from the four groups, the restoration capacity is at least 37-fold higher for H than L chain–donating hybrids.

TABLE I

Fusions Performed with the Sp6/HK Line

Experiment No.	Number of spleens	LPS	Growth per 192 wells	Calculated number of hybrids*	TNP positive	Names of reclones
1	3	–	119/169	206	5	D6-E7
2	1	–	35	37	1	
3	2	+	108	161	12	A1-1; A6-2-1; B3-4-2; E8-2; F4-4; G12-4
4	1	–	12	12	0	
5	1	–	37	40	1	45-3
6	1	–	191	1,010	3	7-2B
7	1	–	58	69	2	1-3
8	1	+	186	666	5	63-34; 61-27
9	1	+	192	~1,010	7	5-1; 6-1; 7-2A; 1-2; 4-3; 8-3
10	1	+	192	~1,010	6	13-4; 14-3
11	1	+	168	420	2	1-C5; 2-E3
12	1	+	132	223	0	
			Totals:	~4,864	44	22

* Calculated using Poisson statistics.

TABLE II

Restoration of the Original Antibody Specificity by the Fusion of H or L Chain–producing Hybridomas with LPS-stimulated Spleen Cells

Fusion line	HL isotype and specificity	Number of hybrids analyzed	Number of hybrids binding			Frequency	Ratio of H/L frequencies
			TNP	SRBC	Cloned		
Sp6/HK	IgM, κ	4,900	44	0	22	1:111	
/LK	TNP	5,900	2	1	0	≤2:5,900	~1:27
Sp2/HK	IgG_{2b}, κ	3,500	NT	24	3	1:145	
/L	SRBC	4,000	2	1	0	≤1:4,000	~1:28
0		900	1	0	0		
Sp1/HK	IgM, κ	670	NT	30	10	1:22	
/L	SRBC	2,400	NT	1	0	≤1:2,400	~1:110
Sp7/HK	IgM, λ_1	1,800	24	0	7	1:75	
/LK	TNP	1,100	0	0	0	≤1:1,100	~1:15
Pooled	L = 1:90 H ≤ 1:3,350			H/L ≤ 1:37			

NT, not tested.

Identification of L Chain Genes by Southern Blotting. To identify particular V_κ genes we analyzed the DNA of the recloned lines by restriction enzyme digestion with the enzymes HindIII, BamHI, and HpaI. These enzymes cut 3′ to the probe and 5′ to the J_κ segments, thus revealing V_κ-J_κ rearrangements. The results of all recloned hybrids are shown in Fig. 1. Sp6/HK DNA shows two κ-specific bands. The band comigrating with the Sp6/HL DNA represents the rearrangement giving rise to the MOPC-21-derived κ chain. Thus, loss of MOPC-21 κ chain expression is not due to the loss of this gene in Sp6/HL. The second band visible in Sp6/HL defines the Sp6-specific V_6-J_5 rearrangement (18). In the Sp6/HK line the V_6-specific band moves to distinct positions in the gel, due to an insertion of an intracisternal A particle (IAP) element into the intron between the amino-terminal leader and the body of the V_6 variable gene (18). This insertion leads to nonexpression of the Sp6-specific κ chain in Sp6/HK. Both Sp6/HK κ bands (indicated in kilobases, Fig. 1) are found in most hybrids made with this line. The remaining bands represent the DNA rearrangements introduced by the spleen cell fusion partner. Half of the 22 lines analyzed for Sp6 have one DNA band comigrating with embryonal DNA (*E*, Fig. 1). In these lines only one band is left for the active V_κ-J rearrangement. Comparing lines with similar restriction patterns indicates the existence of four groups of lines, whose presumptive actively rearranged κ gene is marked by a white dash in Fig. 1.

The largest group, a, includes the lines 5-1, 6-1, 1-C5, 2-E3, and A1-1. Their DNA restriction pattern showed one band comigrating with the Sp6-specific V_κ gene. The second largest group, b, with four members (D6-E7, B3-4, 45-3, 1-3), only differed from group a by giving rise to a slower-migrating band after HpaI digestion. Group c had three members, namely A6-2-1, E8-2, and 61-27. Finally,

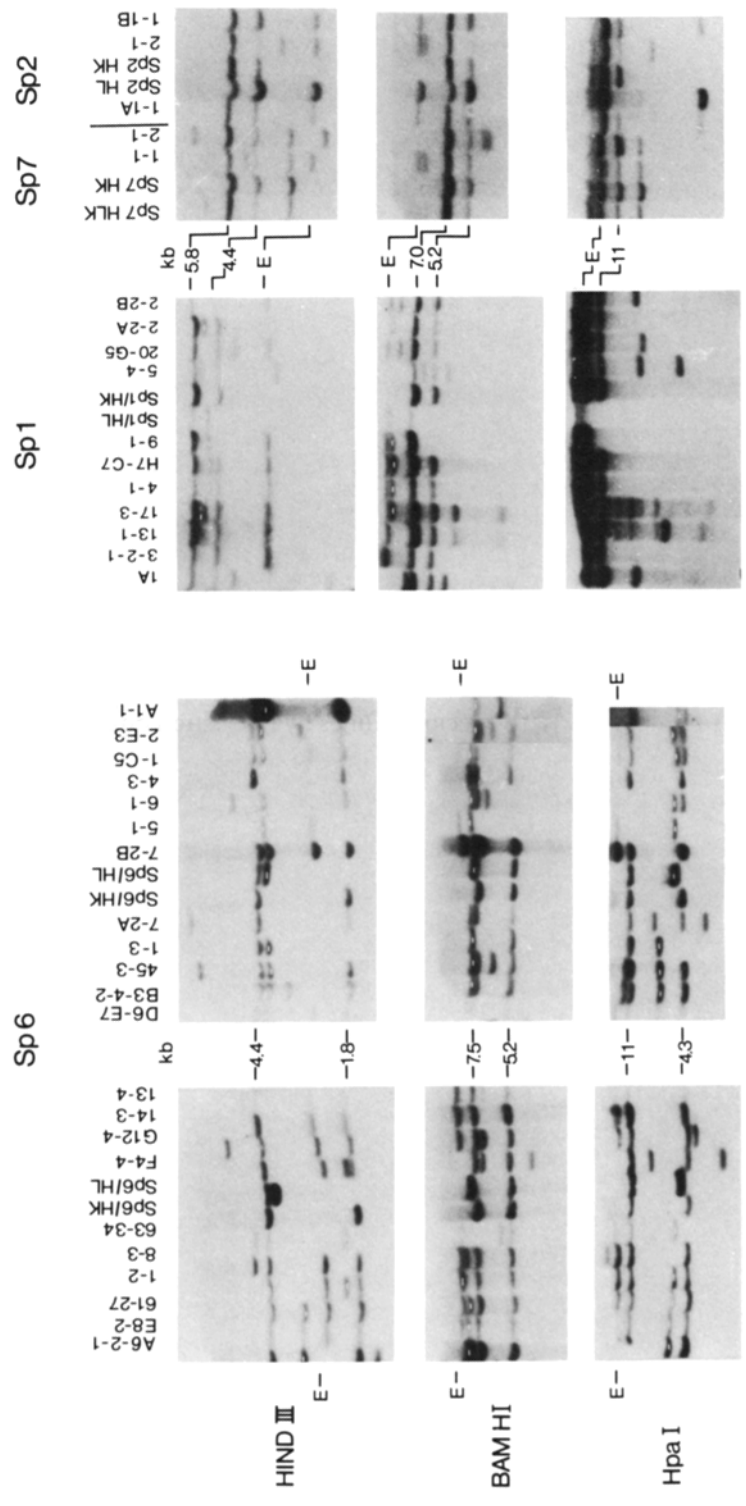


FIGURE 1. Southern blot analysis of κ-encoding DNA. DNA from secondary hybridoma cell lines between Sp6, 1, 7 and 2/HK and BALB/c spleen cells was cut with the restriction enzymes HindIII, BamHI and HpaI. Rearrangement-specific V_κ bands are visualized by using the XbaI-HindIII fragment from the J_κ-C_κ intron. The rearrangements introduced by the Sp/HK fusion partners are indicated in kilobases. The unrearranged fragment is indicated by *E*. Fragments found to comigrate are indicated by white dashes on top of band.

comigration of a V_κ-DNA fragment was also observed for the lines 1-2 and 63-34, defining group d. All other lines show no particular similarities of their restriction enzyme digestion pattern. They constitute a heterogeneous group, e, of six members using different V_κ genes, since clone 14-3 has lost anti-TNP activity and clone 4-3 was shown to use a λ_1 chain. A similar pattern emerged when we analyzed the κ gene rearrangement of 10 hybrids that restored the Sp1/HK line to the original anti-SRBC activity (Fig. 1). Three independent isolates expressed L chain genes indistinguishable in the Southern blot analysis from the L chain gene originally used in the Sp1/HL hybridoma (Fig. 1, clones 4-1, H7-C7, 9-1 [group a]; band marked by white dash). Two other isolates (clones 17-3 and 2-2A [group b]) differed from group a only by a 300 bp size difference of the V_κ gene–specific band after HindIII restriction enzyme digestion of their DNA. They presumably use the same V_κ (Sp1) gene joined to a different J segment. The five other isolates (clone 3-2-1 was unstable and had lost activity) were all different from each other and from groups a and b and thus represent the heterogeneous group c. Clones 20-G5 and 2-2B may use the same V_κ gene segment joined to different J_κ, since a difference of ~300 bp is noted for the actively rearranged band (see also Table IV *C*).

Only three isolates were analyzed by Southern blotting experiments in the Sp2 experiment (Fig. 1). Although the Sp2-specific V_κ band could not unambiguously be identified, two out of the three restriction patterns obtained from the secondary hybridomas were compatible with the idea that they used the same V_κ and J_κ segments used by the original Sp2 L chain (clones 1-1A and 1-1B; see Fig. 1, HpaI digest [lowest band]). The two Sp7 hybrids that were complemented for activity by a κ light chain used different V_κ genes (Fig. 1).

2-D Gel Electrophoresis of the Grouped κ L Chains. To confirm the relation of the grouped κ chains, IEF followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (2-D gels) was performed on purified κ chains. Fig. 2 shows the L chain region of the 2-D gels from the Sp6-derived clones. The relative size of radiolabeled, MOPC-21-derived kappa (κ_{21}) to the L chain of interest was determined in all cases by 1-D SDS-PAGE. Since the position of κ_{21} and κ_{Sp6} were known in 1-D IEF gels it was easy to locate the MOPC-21-derived κ chain in the 2-D gels. In all samples shown (Fig. 2), the κ_{21} chain runs as the left or upper two major dots (the charge difference has been attributed to posttranslational effects (19). The κ_{21} can therefore be used as internal standards in the 2-D gels. The presence of small amounts of κ_{21}-specific dots in lines 5-1 and 6-1 (Fig. 2), which, according to the Southern blots in Fig. 1, have lost the κ_{21} gene, can be explained by the fact that supernatants were obtained 2–3 mo before the DNA extractions were made and that the κ_{21} gene was lost during this time interval.

As can be seen in Fig. 2, members of each group gave rise to identical 2-D patterns of their respective L chains, confirming the results obtained by Southern blotting. Table III summarizes the results obtained with the 20 Sp6/HK clones. Members of group a used the same variable region gene joined to J_5 as the original Sp6/HL hybridoma and their κ chains were indistinguishable from the Sp6 κ chain in the 2-D gel electrophoresis. They were isolated in five independent secondary hybridomas, since hybrids 5-1 and 6-1, as well as 1-C5 and 2-E3, which

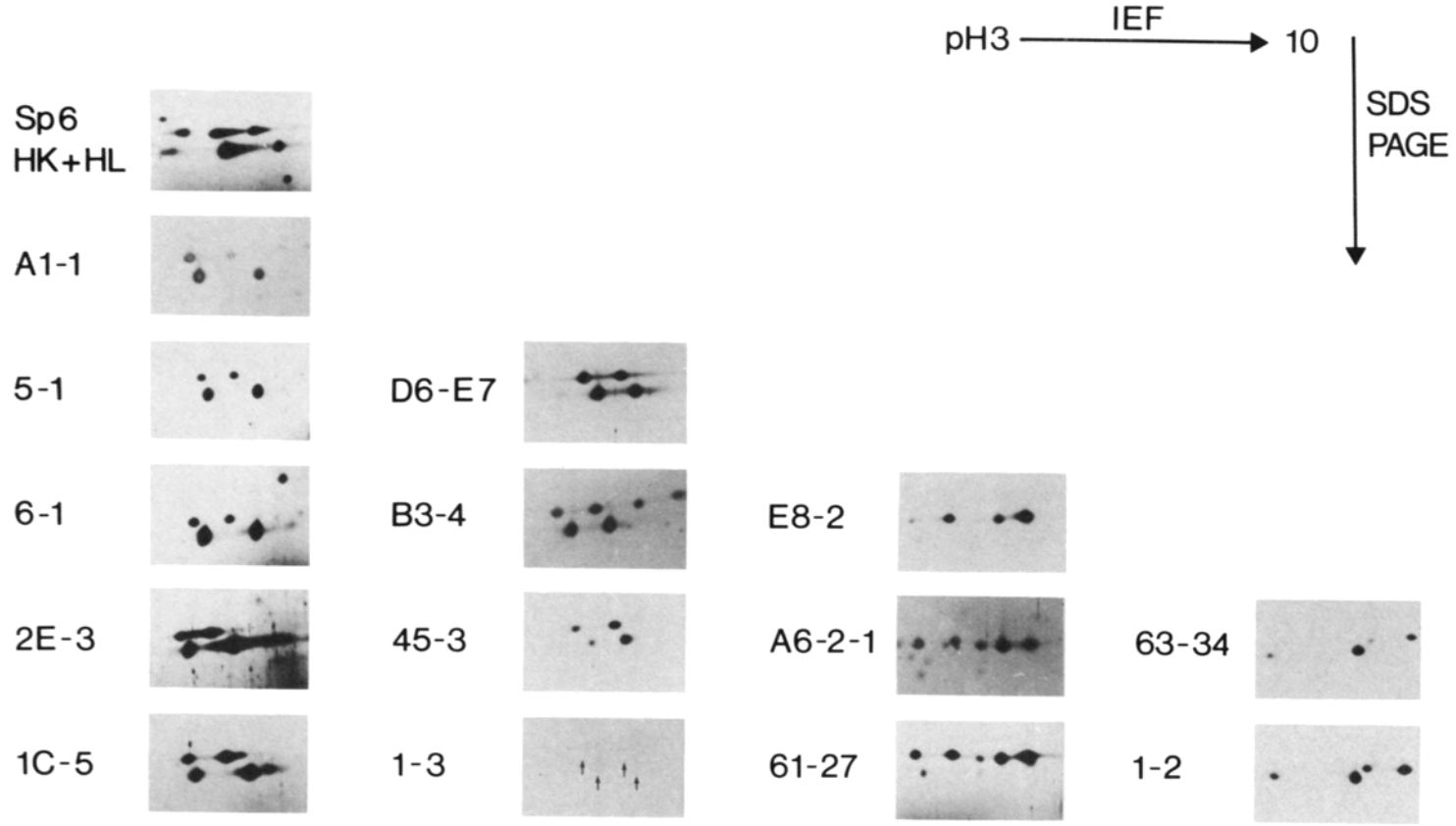


FIGURE 2. 2-D gel electrophoresis of κ chains. Shown are only the κ chain regions of the gels. The gels are grouped according to the Southern results of Fig. 1. The left or upper two dots serve as internal standards and are derived from the MOPC-21 κ chain.

TABLE III

Characterization of the Activity-restoring L Chains

Line	Group/2-D pattern	HpaI*	BamHI	HindIII	Number of isolates	Frequency
		kb	*kb*	*kb*		
Sp6	a/a‡	4, 6	9, 0	3, 8	5	1:490
	b/b	6, 1	9, 0	3, 8	4	
	c/c	5, 1	10, 5	3, 1	3	
	d/d	4, 9	12, 0	2, 0	2	
	e/not a–d	Heterogeneous			6§	
Sp1	a/a‡	~20	11	5, 0	3	1:74
	b/a	~20	11	5, 3	2	
	c/not a	Heterogeneous			5‖	

* The probe used was the XbaI-HindIII fragment from the J_κ-C_κ intron.

‡ The a type is indistinguishable from the original Sp6 or Sp1 L chains.

§ Clone 14-3 had lost anti-TNP activity and showed no specific κ rearrangement (Fig. 1). Clone 4-3 was typed to carry a λ_1 chain and presumably represents a "background" anti-TNP hybrid.

‖ Clone 3-2-1 had lost anti-SRBC activity and showed no specific κ rearrangement (Fig. 1).

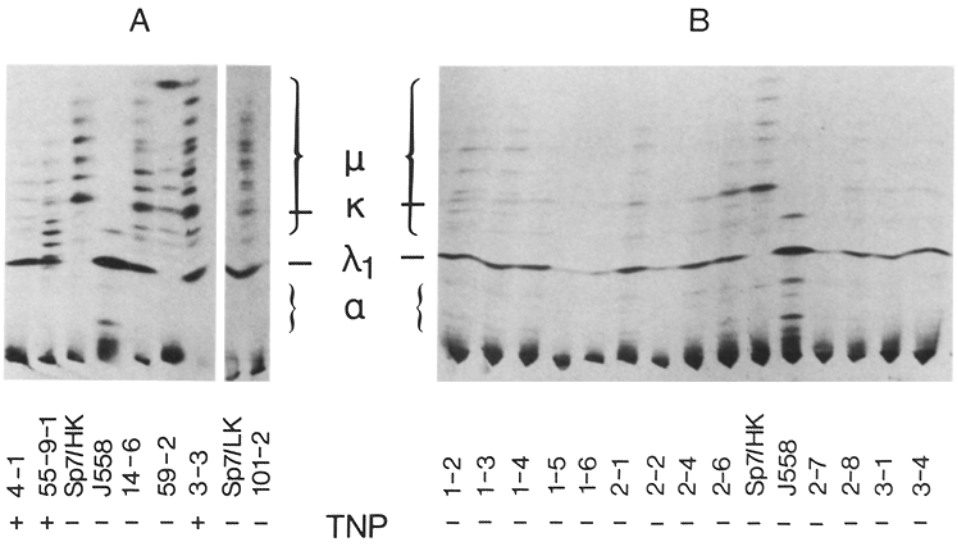
originated from fusions 9 and 11, respectively (Table I), showed a different restriction pattern of the silent κ chain allele (Fig. 1). For Sp1, similar 2-D L chain spots were seen for the groups a and b (Table III; gels not shown) that were indistinguishable from the Sp1 original L chain, whereas spots different from pattern a were found in the heterogeneous group (c).

The original $V_\kappa 6$ and $V_\kappa 1$ genes were therefore the most frequent ones recovered from the LPS-stimulated spleen cell pool. They were made by approximately every 490th and 74th spleen cell that was hybridized.

It is possible that the repeatedly isolated κ genes are identical (or similar) to their unmutated germline configuration, whereas those isolated only once needed somatic changes to be able to restore antibody specificity. We therefore analyzed in more detail the hybrids obtained with the line Sp7/HK, which can be complemented by the λ_1 gene product and where the germline-encoded chain is known to be expressed in the J558 myeloma line (20).

Evidence for λ_1 Chain Heterogeneity in the LPS-stimulated Spleen Cell Population. 7 out of 24 TNP-restoring secondary Sp7/HK hybridomas were recloned (Table II). They were typed in radioimmunoassays using anti-mouse κ-, λ-, and λ_1-specific monoclonal rat antibodies. Two expressed κ chains, one was typed λ_2 or λ_3, and four were identified as λ_1 secretors.

About 5% of all Sp7/HK hybrids made λ chains and about half of those were typed to secrete λ_1 chains. Of 15 λ_1-positive supernatants obtained in one fusion, only 5 were found also to exhibit anti-TNP specificity. Of those, two λ_1^+, TNP^+ lines (55-9-1 and 3-3) and two λ_1^+, TNP^- lines (14-6 and 101-2) were recloned (see below). This heterogeneity indicates that either 10 out of 15 hybrids had lost the Sp7-derived μ heavy chain or that the λ_1 gene product itself was heterogeneous. IEF electrophoresis was performed on radiolabeled culture supernatants of three recloned λ_1^+, TNP^+ Sp7/HK hybrids (4-1, 55-9-1, 3-3 [Fig.



3*A*]), two λ_1^+, TNP$^-$ lines (14-6, 101-2), and an unrelated κ^+, TNP$^-$ line (59-2). J558-labeled supernatant was run to indicate the position of the germline λ_1 product.

It can be seen (Fig. 3 *A*) that all λ_1 chains, including the Sp7-derived one (Sp7/LK), comigrated with the J558-derived λ_1 chain. λ chain heterogeneity was therefore not revealed at this level but must exist since the two negative lines showed the presence of two H chains, with a series of bands comigrating with Sp7 μ.

To prove directly whether or not the germline λ_1 chain could complement the Sp7 H chain for anti-TNP activity, the J558 myeloma line was fused to Sp7/HK. From 18 independent hybrids, none showed anti-TNP activity, although both the Sp7 μ chain and the J558 λ_1 chain were coexpressed (reclones of the hybrids 1, 2, and 3 are shown in Fig. 3*B*). We conclude that about one-third of the λ_1 chains of LPS-stimulated spleen cells are non-germline but carry mutation(s) that allow the restoration of the original Sp7 anti-TNP activity.

Use of Particular V Genes in Different Complementation Groups. We compared the spleen cell–derived rearranged V_κ bands of Fig. 1 of each complementation group with each other. Samples showing presumptively identical V_κ bands were run next to each other and gave the results summarized in Table IV. DNA of the Sp6 8-3 clone (group e) contained a sequence that comigrated with the one from the Sp1/2-2 A clone (group b), with all four restriction enzymes used. Identity was also observed for the original Sp1/HL light chain and the one used in Sp7 clone 1-1. The first pair differed from the second only by a small change in the size of the actively rearranged band, which may be due to the use of the same V region with different J segments. A similar size-shift was observed for the pair, Sp6 group e, clone E8-2 and Sp2 clone 2-1, so that here again the same V could have been joined to different J segments.

TABLE IV
Similar Restriction Pattern of κ Variable Genes in Different Complementation Groups

	Line/group	Clone	HpaI	BamHI	HindIII	XmnI
				kb		
A	Sp6/3	8-3	≲20	11	5.0	7.6
	Sp1/b*	2-2A	≲20	11	5.0	7.6
	Sp1/a*	Sp1/HL	≲20	10.5	4.8	7.3
	Sp7	1-1	≲20	10.5	4.8	7.3
B	Sp6/c	E8-2	5.2	10.5	3.3	5.1
	Sp2	2-1	5.4	11	3.5	5.4
C	Sp1/c	20-G5	5.0	9.3	3.6	4.8
	Sp1/c	2-2B	5.5	9.5	3.9	5.2

* Size differences of restriction fragments as compared with those in Table III reflect the variability of two independent determinations.

The radiolabeled supernatants of those lines (Table IV, *A* and *B*) were run on IEF-PAGE and a comigrating L chain band different from the MOPC-21 κ chain was identified for the four lines in Table IV *A* and another one for the two in Table IV *B* (data not shown). We conclude that certain V_κ regions contribute to different specificities.

Discussion

Different L Chains Restore One Activity. We have generated a set of secondary hybridomas that express a variety of L chains together with one H chain and one specificity. Restoration was achieved for two different specificities (TNP and SRBC) with two different Ig classes (IgM and IgG2b) and was independent of the class of L chain originally used for antibody specificity (κ, λ). We conclude that restoration of antibody activity with a set of spleen cell–derived L chains and a given H chain is a general phenomenon. The frequencies varied from 1 in 22 to 1 in 145, with a mean value of 1 in 90 (Table II). 10 different V_κ genes, each joined to a particular J_κ, were able to restore the Sp6 H chain to its original anti-TNP activity. Similarly, five different V_κ genes restored the anti-SRBC activity of Sp1. From the three reclones analyzed for Sp2, at least two different L chains could restore the anti-SRBC activity. The Sp7 line, which originally used the λ_1 chain for its anti-TNP activity, was restored not only by λ_1 chains but also by λ_2 or λ_3 chains and by two different κ chains.

The use of anti-TNP and anti-SRBC specificity was dictated by the cell lines that were available, and might be criticized. The hapten TNP may require only minimal complementarity because it is too small to fill the antibody combining site. Complementation of H chains for anti-TNP activity with spleen cell–derived L chains could therefore be particularly easy and not representative. The fact that two monoclonal antibodies recognizing two different determinants on SRBC exhibited similar complementation frequencies argues against such a possibility (Table II). Preliminary experiments using an antiphosphorylcholine-specific hy-

bridoma line indicate a complementation frequency of ~1 in 20. Nevertheless, more hybridomas with different specificities must be analyzed to confirm the surprisingly high complementation frequencies observed.

It is difficult to evaluate the quality of the restored activities. The secondary hybridomas synthesize and assemble two H and two L chains. A whole array of hybrid molecules are therefore generated, especially in the Sp1, Sp6, and Sp7 hybrids where in most of the cases two IgM molecules intermingle. Preferential H–L chain associations may further complicate the issue (21, 22). Both effects may result in reduced apparent agglutination titers as compared with the pure active antibody. Table V summarizes the direct and indirect agglutination titers obtained from supernatants of lines collected at the time the cells were frozen away. Generally, none of the secondary hybridomas gave titers higher than the original parental line, although these titers were reached with a variety of lines, especially by members of group a that used the same V_κ-J_κ combinations as the parental lines. The influence of affinity on agglutination titers was evaluated in a comparison of Sp6 and igx 2.15, a variant line derived from it (14). In a phage inactivation inhibition assay using TNP-caproic acid as inhibitor, Sp6 IgM was found to have an affinity of 10^{-4} M, and igx 2.15, a 10-fold lower affinity. When equal amounts of purified Sp6 and igx 2.15 IgM were titrated in the agglutination assay, a drop in titer of ~30-fold (3^3) was found (14). Such a severe drop in titer was not observed in any of the secondary hybridomas. We conclude, therefore, that the restored hybridomas have equal or only slightly reduced affinities compared with the original antibodies and that no clear case of a better quality antibody was observed.

Nevertheless, these lines will be useful to study the structural requirements imposed on L chains when a given antigen-binding activity has to be maintained together with one particular H chain. Here we have used the set of secondary hybridomas to answer questions about the expressed repertoire of H and L chains in the mouse. Antibody affinity and fine specificity remain of secondary importance in such a study.

The Expressed Repertoire Is as Diverse as the One Predicted from Random Assembly of Variable Gene Segments. Although activity restoration of H chains with spleen cell–derived L chains was achieved in about every 90th hybrid, we have no evidence that the reverse occurred. Restoration of L chains with spleen cell–derived H chains was too rare to be distinguished from background specificities (Table II). The frequencies for L chain restoration with spleen cell–derived H chains are therefore overestimates. The data in Table II indicate that it was at least 37-fold rarer to find an activity-restoring H chain than L chain in the spleen cell pool. We consider this difference to be due to an at least 37-fold greater heterogeneity of H over L chains.

The potential repertoire of genes from Southern blotting experiments can be calculated as about 160 $V_H \times 12\ D_H \times 4\ J_H = 8{,}000\ VDJ_H$ genes and about 90 $V_L \times 4\ J_L = 360\ VJ_L$ possible genes. Thus, 8,000 ÷ 360 or ~20-fold more H than L chains could be made. Our results confirm an at least 20-fold greater heterogeneity of H over L chains in the expressed repertoire. Assuming equal secondary heterogeneity in the V and J segments due to somatic and joining processes for H and L chains, the observed minimum H/L difference of 37-fold, corrected

TABLE V

Hemagglutination Titers of Supernatants from Secondary Hybridoma Lines

Line	Hemagglutination titers (3^n)											
	n equals: TNP-SRBC		Line	*n* equals: TNP-SRBC		Line	*n* equals: SRBC		Line	*n* equals: SRBC	
	D*	I*		D	I		D	I		D	I
Sp6/HL	2	6	Sp7/HLK	2	7	Sp1/HL	2	5	Sp2/HL	—	>8
Sp6/HK	—	—	Sp7/HK	—	—	Sp1/HK	—	—	Sp2/HK	—	—
Sp6/L	—	—	Sp7/L	—	—	Sp1/L	—	—	Sp2/L	—	—
A1/1	2	6	1-1 (κ)‡	3	7	9-1	1	5	1-1	—	5
5-1	2	6	2-1 (κ)‡	0	6	4-1 (a)	2	3	2-1	ND	
6-1 (a)§	2	6				H7-C7	2	4			
2E-3	2	7	4-1	2	7						
1C-5	2	6	55-6 (λ_1)	2	4	2-2A (b)	2	4			
			3-3 (λ_1)	2	7	17-3 (b)	2	4			
D6-E7	2	5	1-15	2	7						
B3-4-2 (b)	3	6				13-1	2	4			
45-3 (b)	0	3	3-1 ($\lambda_{2 \text{ or } 3}$)	0	5	5-4	2	3			
1-3	2	5				20-G5 (c)	2	4			
			14-6 (λ_1)	—	—	2-2B	1	3			
E8-2	1	3	101-2 (λ_1)	—	—	1-A	2	5			
A6-2-1 (c)	1	4									
61-27	2	7									
63-34 (d)	0	3									
1-2 (d)	2	7									
7-2 A	0	3									
F4-4	1	3									
7-2 B	2	7									
G12-4 (e)	1	4									
4-3	1	4									
13-4	1	5									
8-3	2	7									

* D, direct agglutination; I, indirect agglutination, using a rat monoclonal anti-mouse $C_\mu 4$ antibody to enhance agglutination, except for the Sp2/HL tests, where a rabbit anti-mouse IgG antiserum was used.

‡ Class of L chain conferring specificity; the λ_1 of lines Sp7/14-6 and 101-2 may represent the germline configuration, which cannot complement (see text). All other lines, with the exception of Sp6/4-3 (λ_1), use κ chains for complementation.

§ a–e in parentheses refer to groups (see Table III).

for the V_H/V_L ratio ($160 \div 90 = 1.8$), gives a value of at least $37 \div 1.8 = 20$-fold attributable to the D segments of H chains. This value is high enough to allow random V_H-D_H-J_H joining and leaves room to accommodate more D segments (21) and high D-joining variability (5).

All L Chains But Only a Subset of H Chains Can Make Anti-TNP Antibodies. A "suitable" chain is defined as a chain that can form an anti-TNP molecule, provided it has a "fitting" partner chain. We termed the probability of picking,

by random fusion, a suitable L chain, P_{sL}; the probability of picking (when the suitable H chain is given) a fitting L chain, P_{fL}; the probability of randomly picking a suitable H chain, P_{sH}; and the probability of picking (when the suitable L chain is given) a fitting H chain, P_{fH}.

About 1:2,000 of randomly picked cells make anti-TNP antibodies (Table II, Sp2/L). This can be looked at in two ways: (*a*) The random cell may have a suitable H chain and a fitting L chain; therefore, anti-TNP frequency $\fallingdotseq P_{sH} \times P_{fL}$ = 1:2,000. (*b*) The random cell may have a suitable L chain and a fitting H chain; therefore, anti-TNP frequency = $P_{sL} \times P_{fH}$ = 1:2,000. Since P_{fL} was found to be 1:100 (Table II, Sp6/HK and Sp7/HK), it follows from *a* that P_{sH} becomes 1:20. That means that every 20th randomly picked H chain is suitable to form an anti-TNP antibody.

Since we found no evidence that cells making fitting H chains (to given suitable L chains; Table II, Sp6/LK, Sp7/LK) are more frequent than the randomly picked cells making anti-TNP, we conclude that $P_{fH} \leq$1:2,000. Inserting this value into equation *b* results in $P_{sL} \simeq 1$, that is to say, *all* L chains are suitable (provided they combine with the right H chain) to form an anti-TNP antibody. In general terms, screening through the H chain repertoire with any given L chain will give rise to any antigen specificity. Screening through the L chain repertoire with any given H chain will generate the desired antibody specificity only in every 20th case.

The Original L Chain Gene Is Rescued Most Frequently. When the cloned hybrids were grouped according to their DNA restriction pattern of the V_L region and their 2-D gel electrophoresis pattern, it became apparent that the L chains originally used in the Sp6 and Sp1 hybridoma cells were represented in the largest groups, with 5 out of 21 (Sp6) and 3 out of 10 (Sp1) cases (Table III). From the seven reclones analyzed for the restoration of the Sp7 activity, four used the original λ_1 gene, which also represents the largest group. Since Sp1, 6, and 7 were hybridoma lines obtained from immunized mice, it seems that with a given H chain only the frequency of L chain determines participation in an immune response.

Pool Sizes of L Chains Differ Considerably. From the two largest groups (Sp6 and Sp1 [group a], Table III) the frequency of Sp6 L chains was calculated to be 5 of 21, 10 of 44, 10 of 4,900 hybrids, or 1 of 490. A similar calculation for Sp1 gave a value of 1 in 74. Thus, the Sp1 V_κ region is expressed seven times more frequently than the Sp6 V_κ region. This could be due to the possibility that the Sp1 κ chain uses a germline gene and the Sp6 κ chain has to be mutated to complement activity. Another possibility is that the Sp1 V_κ is joined to J1 or J2, which were shown in LPS-stimulated spleen cells to be rearranged 5–10 times more frequently than J4 and J5 (C. Coleclough, personal communication). We favor the latter idea since indeed Sp6 V_κ is joined to J5 and since the Sp6 hybridoma was derived from a mouse after a single immunization with TNP-LPS (7), making germline expression of V_κ6 more likely.

If the V_κ6-J5 joining occurs as frequently as the V_κ6-J4 joining and both together are 5–10 times less frequent than the presumptive V_κ6-J1 and -J2 joining, then ~10 of 4,900 spleen cells use V_κ6-J5 (Tables II and III), 20 of 4,900 use V_κ6-J4 and -J5, and 100–200 of 4,900 use V_κ6 joined to J1, 2, 4, 5.

That means that every 25th to 50th spleen cell uses $V_\kappa 6$. If $V_\kappa 1$ is indeed joined to J1 and J2 (Table III, Sp1 groups a and b), it is used in about every 45th spleen cell. For comparison the λ_1 V gene is used in about every 40th spleen cell. These considerations raise the possibility that only ~40 different V_κ segments participate in the expressed L repertoire.

Frequent Expression of Non-germline λ_1 and κ Chains. Fusion of the Sp7/HK line with J558 myeloma cells resulted in 18 independent hybrids expressing the Sp7 μ chain and the J558 λ_1 chain. None of these hybrids showed anti-TNP activity. Since the J558 λ_1 chain represents the germline-encoded form, we conclude that Sp7 can be complemented for anti-TNP activity only by a non-germline λ_1 chain. This chain was found to have an unaltered isoelectric point as compared with the J558 λ_1 (Fig. 3). Its frequency was one out of three λ_1 chains in the LPS pool. Thus, at least one-third of the λ_1 chains cannot be in the germline configuration. It is tempting to speculate that such a high frequency might be due to a V-J joining variation.

Comparing the use of certain V_κ genes, we found that the most frequently obtained V_1-J_a combination (Sp1 group a, frequency 1:74, Table III) was used once to restore the Sp7 anti-TNP activity in clone 1-1 and the second most frequently obtained V_1-J_b combination (Sp1 group b), frequency 1:110, using the same V region as Sp1 group a) was found once in the Sp6 anti-TNP activity–restoring clone 8-3 (Table IV). The frequency of V_1-J_a and V_1-J_b restoring the Sp7 and Sp6 antibody activity is 5- and 30-fold lower than the frequency found for the restoration of the Sp1 activity.

We conclude that for κ genes also, variants are found in the LPS pool. The variants were found in the heterogeneous group (Table III, Sp6 [group e], Sp7 κ group) and may indicate that most of these rarely obtained V_κ genes may need mutation(s) to be able to restore the Sp6 or Sp7 antibody activities. Thus, about one-third (the heterogeneous groups) of the κ chains obtained may represent variant, non-germline κ chains.

Summary

Variants of four hybridoma lines secreting antibodies specific for either trinitrophenyl (TNP) or sheep erythrocytes (SRBC) were isolated which have lost either the specific heavy (H) chain or the specific light (L) chain. They were fused to lipopolysaccharide-stimulated mouse spleen cells and the resulting secondary hybridomas were screened for the restoration of the original antibody specificity. Antibody activity was 37 times more frequently restored with fusion lines donating H chain than with those donating L chain.

We obtained a variety of different (spleen cell derived) L chains in association with one H chain and one specificity. We found that those L chains originally associated with a given H chain were rescued most often. Their frequencies were 1:74 and 1:490 for an anti-SRBC– and an anti-TNP–restoring κ L chain, respectively. Two most commonly observed V-J_κ combinations in one anti-SRBC complementation group were detected with a 5- and 30-fold reduced frequency in two anti-TNP groups, indicating somatic diversification of κ chains. It is shown that the Sp7/HK line resumes anti-TNP activity with a mutated, non-germline λ_1 chain, which was found in 30% of λ_1-expressing hybridomas.

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