17. ddC-resistant viruses that contained the K65R substitution alone could be selected for resistance to the other drugs, suggesting that the inability to outgrow into a drug-resistant virus population is not a common property of all nucleoside analog–resistant viruses.
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19. The sequence of the template oligonucleotide was as follows: 5′-GTGGAAAATCTCTAGCAGTGGCGCCCGAACAGGGACCTGAAAGCG-3′. The four primers that were used to study nucleotide insertion opposite A, C, G, and T, respectively, were as follows: 5′-CGCTTTCAGGTCCCTGTTCGGGCGCCAC-3′ (PBS-A), 5′-CGCTTTCAGGTCCCTGTTCGGGCGCCACT-3′ (PBS-C), 5′-CGCTTTCAGGTCCCTGTTCGGGCGCCA-3′ (PBS-G), and 5′-CGCTTTCAGGTCCCTGTTCGGGCGCCACTGCT-3′ (PBS-T).
20. Before use, crude oligonucleotides were resolved on 12 to 20% polyacrylamide-urea gels (Sequagel, National Diagnostics) and the major band eluted in buffer containing 0.5 mM ammonium acetate, 0.1% SDS, and 10 mM magnesium acetate and purified on a Sep-Pak column (Millipore). The purified oligonucleotides were resuspended in 10 mM tris-Cl and 1 mM EDTA (pH 8.0). The ends of the primer oligonucleotides were labeled in a forward reaction with [γ-^{32}P]adenosine triphosphate (3000 Ci/mmol) and T4 polynucleotide kinase (40 U) (New England Biolabs). The template and primer were annealed with a slight excess of template (1.27:1) to ensure that the primer was annealed completely. ^{32}P-labeled primer (74 pmol) was mixed with 94 pmol of template in 50 mM tris-Cl, pH 8.0; acetylated bovine serum albumin (50 μg/ml); and 2 mM β-mercaptoethanol in a total volume of 1 ml, placed in boiling water for 3 min, and allowed to cool slowly to room temperature over 1 hour.
21. Insertion events can be measured in the two distinct phases of DNA polymerization: the initial extension (standing start) and the subsequent synthesis (running start). In standing starts, the reactions are performed in the presence of only one dNTP at a time to measure the insertion opposite the first unpaired template base. Running-start reactions, on the other hand, are intended to measure the insertion at a target base at least two bases beyond the primer 3′-terminus.
22. The preparation of purified heterodimer RT from HxB2 (wild type) (*35*) was as described previously and that of HxB2 containing the M184V alteration was by means of high-performance liquid chromatography after separate expression of p66 and p51 subunits. The preparations were nuclease-free and had a specific activity of 800 and 100 units per milligram of protein, respectively [one unit is defined as 1 nmol of dTTP incorporated into poly(rA)oligo(dT) in 10 min at 37°C].
23. We initiated the misinsertion reactions by combining equal volumes (5 μl) of solutions A (enzyme and template-primer) and B (dNTP and salts). Solution A was made by diluting the enzyme in ice-cold template-primer to enzyme concentrations of 0.21, 4.2, or 36 nM, which corresponded to enzyme:template-primer molar ratios of 1:170, 1:8.5, and 1:1, respectively (in general, an excess of template-primer was used to ensure that all enzyme molecules were in a bound state). Solution B contained the dNTP at a concentration of 0 to 10 mM in 160 mM KCl; 100 mM tris-Cl, pH 8.0; 20 mM dithiothreitol, and 12 mM $MgCl_2$. A series of reactions was carried out at 37°C with increasing concentrations of dNTP for an empirically determined reaction time to allow the conversion of about 25% of primer to extension product. Reactions were terminated by the addition of 30 μl of stop solution (95% formamide and 20 mM EDTA). The boiled terminated reaction (2 to 8 μl) was loaded onto a 12 to 18% polyacrylamide-urea gel and electrophoresed for 1.75 to 2.5 hours at 30 W. Gels were then autoradiographed within the linear-response range of the film. The products included not only primers extended by one nucleotide (n+1), but in some instances those that were the result of a mispair extension. An important concern in this type of experiment is whether the primer extensions were due to contaminating correct dNTP. This problem is particularly common while studying the insertion of dCTP opposite template base A, because dCTP preparations accumulate dUTP generated by spontaneous deamination. Efforts were made to ensure the absence of such contaminating correct dNTPs in our experiments. First, all the dNTPs were commercial preparations that were HPLC-analyzed. No traces of correct dNTPs were present as reported by the supplier. If any contaminating correct dNTPs were present, they would be at subnanomolar concentrations that would poorly catalyze the insertion reaction. Second, at high resolution, misinsertion products often display altered mobilities when compared to products resulting from correct insertion. We find that nearly all types of misinsertions displayed altered mobilities. Most importantly, with regard to AC mispair formation, a definite difference was seen between the mobility of dCTP-extended PBS–A primer and dTTP-extended PBS–A primer, strongly suggesting that the dCTP-misinsertion and not dUTP (possibly derived from deamination) insertion was examined.
24. Analysis of reaction products involved scanning the autoradiograms with a Molecular Dynamics densitometer (Model 300A), followed by determining the intensities of product bands with the program ImageQuant (Molecular Dynamics). The relative amounts of unextended (I_U) and extended (I_E) primers were evaluated for each reaction. The initial velocities of the product (I_E) formation (relative V_E) were determined according to the procedure described by Goodman and colleagues (*18*). The relative V_E values from each set of reactions were used to calculate relative V_{max} and K_m for the formation of that mispair by plotting reciprocal initial relative velocities (1/relative V_E) against reciprocal variable dNTP concentrations (1/[dNTP]) and fitting the data to the appropriate rate equations with the FORTRAN programs of Cleland (*36*). The enzyme concentration factor was taken into consideration when calculating V_{max} because variable amounts of the enzyme were used depending on the variation in the rates of formation of different mispairs. The efficiency of misinsertion for that mispair, f_{ins}, was then derived as described by Goodman and colleagues (*18*).
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32. This is confirmed by studies showing that ratios of tissue culture infective dose [$(TCID)_{50}$] to p24 were at least four times higher in culture fluids of peripheral blood mononuclear cells or MT-4 cells infected by the M184V clone of infectious HIV than in cells infected by the HxB2 clone of HIV or the HIV_{IIIB} isolate.
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Somatic Mutation of Immunoglobulin V Genes in Vitro

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The molecular mechanism behind affinity maturation is the introduction of point mutations in immunoglobulin (Ig) V genes, followed by the selective proliferation of B cells expressing mutants with increased affinity for antigen. An in vitro culture system was developed in which somatic hypermutation of Ig V genes was sustained in primed B cells. Cognate T cell help and cross-linking of the surface Ig were required, whereas the addition of lipopolysaccharide or a CD40 ligand to drive proliferation was insufficient. This system should facilitate understanding of the molecular and cellular mechanisms that regulate somatic mutation and B cell selection.

The process of somatic hypermutation of Ig V genes within a population of proliferating memory B cell precursors provides a pool of cells that are subject to selection for increased affinity for antigen; this is the basis of affinity maturation of antibody responses (*1–4*). Hypermutation and selection occur specifically within germinal centers in B cell follicles (*5*, *6*), but the molecular mechanism behind somatic mutation has remained elusive, to a large extent because of the absence of well-defined in vitro models (*7*). An in vitro model should also facilitate analysis of the extra- and intracellular signals regulating this mechanism and of the regulation of the various fates of B cells that participate in immune responses.

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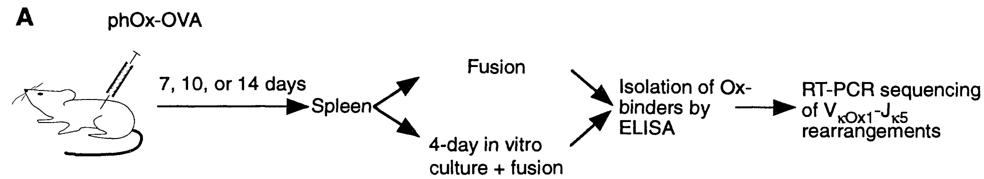
Fig. 1. (**A**) Schematic illustration of the experimental strategy. (**B**) Summary of sequences from PCR-amplified $V_{\kappa Ox1}$ mRNA from hybridomas made at days 7, 10, and 14 after immunization (*15*). Also shown is a summary of sequence data from day 7 and day 10 in vitro cultures stimulated with T_H cells plus anti-κ–Sepharose (anti-κ–S) or with LPS plus anti-κ–Sepharose, as well as data from day 10 in vitro cultures where primed B cells were stimulated with CD40L plus anti-κ–Sepharose or with T_H cells only (T_H2) (*15, 16*). The sequence data in Fig. 2 are also summarized; the data are from randomly selected phOx-binding hybridomas from two independent experiments with pooled B cells from three mice [see also (*2, 5, 9*)]. The *P* values were derived by Fischer's exact test using the frequency of mutation at the day of initiation of culture as a reference (days 7 and 10, respectively).

A



B

Hybridoma source	Number of sequences	Number of mutated sequences	Number of mutations	Mutations per mutated sequence	Frequency of mutations	*P*
Day 7						
Ex vivo	4	0	0	0	–	–
LPS + anti-κ–S	6	2	2	1	1/855	>0.05
T_H2 + anti-κ–S	20	1	1	1	1/5700	>0.05
Day 10						
Ex vivo	15	5	10	2.0	1/428	–
LPS + anti-κ–S	22	10	16	1.6	1/392	>0.05
T_H2 + anti-κ–S	25	21	60	2.9	1/118	0.002
T_H2	7	2	4	2.0	1/499	>0.05
CD40L + anti-κ–S	6	0	0	0	–	>0.05
Day 14						
Ex vivo	8	6	16	2.7	1/142	–

Fig. 2. Sequences from hybridomas made from splenic B cells isolated 10 days after immunization with phOx-OVA and restimulated in vitro for 4 days with LPS plus anti-κ–Sepharose or with T_H2 cells plus anti-κ–Sepharose (*15*). The top sequence is the germline gene of $V_{\kappa Ox1}$ with the amino acid translation shown above [numbered according to Kabat (*17*)]. Complementarity-determining regions (CDRs) are boxed; only codons containing mutations are shown. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

									CDR1											CDR2		
	3	6	10	15	16	18	19	20	21	25	26	29	31	32	34	39	42	45	47	49	52	53	55
	V	Q	I	P	G	K	V	T	M	A	S	S	S	Y	H	K	T	K	W	Y	S	K	A
H3	GTT	CAG	ATC	CCA	GGG	AAG	GTC	ACC	ATG	GCC	AGC	AGT	AGT	TAC	CAC	AAG	ACC	AAA	TGG	TAT	TCC	AAA	GCT
Day 10 LPS + anti-κ–Sepharose																							
15-164-61	---	-T-	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	CC-	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	C--
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
15-175-38	---	---	---	---	A--	---	---	---	---	---	---	---	---	---	---	---	--T	---	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	G--	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
33-10-11	---	---	---	---	---	---	---	---	---	---	--T	---	G--	---	---	---	---	---	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
33-10-	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	G--	---	---	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Day 10 T_H2 + anti-κ–Sepharose																							
15-175-43	---	---	--A	---	---	-G-	---	C--	T--	---	---	---	---	--T	---	---	---	CC-	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	CC-	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-A-
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	G--	G--	---	---	---	---	---	---	---	---	---	---
15-164-35	---	---	---	---	---	---	---	---	---	---	---	---	---	--T	---	---	---	---	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	CC-	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
15-175-38	---	---	---	---	---	---	-C-	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
15-175-138	--G	---	--A	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
15-175-	---	---	---	---	---	---	---	---	---	---	---	G--	T--	---	---	---	---	---	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-C-	---	---	---	---	-T-	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
33-09-22	---	---	---	---	CT-	---	---	---	---	---	---	---	---	---	---	---	---	---	A--	---	---	---	---
33-09-33	---	---	---	---	---	---	---	---	---	T--	---	G--	---	---	---	---	---	---	---	---	---	---	---
33-09-35	---	---	---	---	---	---	---	---	---	T--	---	---	---	---	---	---	---	---	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	CT-	---	---
33-10-46	---	---	---	---	---	---	---	---	---	---	---	G--	C--	---	---	---	---	---	---	---	---	---	---
15-75-23-H10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
33-10-48	---	---	---	---	---	---	---	---	---	---	---	---	T--	---	---	---	---	---	---	---	---	---	---

We reasoned that an attempt to investigate somatic mutation in vitro should mimic as closely as possible the kinetics and conditions for the same event in vivo. To this end, we isolated spleen cells from BALB/c mice that had been immunized with 2-phenyloxazolone coupled to an ovalbumin carrier (phOx-OVA). Half of the cells were fused with SP2/0 cells to generate hybridomas, and the rest were set up in culture under various conditions (Fig. 1A). The response to phOx is well characterized; most B cells use a particular V_H-V_L combination in the primary response [V_{HOx1} and $V_{\kappa Ox1}$ (2, 4)], although the pattern of mutations differs with the carrier used (8). The expressed $V_{\kappa Ox1}$ genes from phOx-binding hybridomas were sequenced by the reverse transcription polymerase chain reaction (RT-PCR); to avoid bias from Taq polymerase–induced mutations, we omitted cloning steps. $V_{\kappa Ox1}$ sequences (rearranged to $J_{\kappa 5}$) obtained from phOx-binding hybridomas derived 7 days after immunization showed very few or no mutations. At day 10, 5 of 15 sequences contained mutations, and the frequency of mutation was 1/428 bases sequenced (Fig. 1B). At day 14, the frequency of mutation had risen to 1/142 bases sequenced, and six of eight sequences were mutated. These kinetics agree with those found by others (2, 4) and indicate an onset of mutation in the spleen from 7 to 10 days after immunization.

Purified B cells from mice immunized 7 or 10 days previously were cultured for 4 days with lipopolysaccharide (LPS) in the presence or absence of antibodies to κ chain (anti-κ) coupled to Sepharose. At the end of this period, cells were fused to SP2/0 and screened for secretion of phOx-binding antibodies. LPS stimulation, which was previously shown not to induce somatic mutation (7), also failed to maintain hypermutation when combined with signals from surface Ig (sIg) (anti-κ–Sepharose). Whether the cultures were started at day 7 (Fig. 1B) or day 10 (Figs. 1B and 2), we could detect no significant increase in the frequency of somatic mutation over that observed in the ex vivo hybridomas derived at these time points. Thus, proliferation plus sIg cross-linking—as might be mediated in germinal centers by antigen on follicular dendritic cells (FDCs) (9)—is insufficient to maintain mutation.

Given that somatic hypermutation is a feature of T cell–dependent antibody responses, we next investigated the capacity of T helper (T_H) cells to maintain mutation in vitro. When B cells from spleens 7 days after immunization were cocultured for 4 days with an alloreactive T_H2 clone [secreting interleukin (IL)-3, IL-4, and IL-5 (10)] plus anti-κ–Sepharose, no increase in mutation was detected relative to ex vivo cells (Fig. 1B). However, when B cells isolated from mice 10 days after immunization were cocultured for 4 days with the alloreactive T_H2 clone plus anti-κ–Sepharose, there was an increase in the number of mutations observed relative to the day 10 ex vivo hybridomas (frequency of mutation, 1/118 versus 1/428; $P = 0.002$), which compared favorably with the frequency of mutation that was observed in ex vivo cells at day 14. These sequences are shown in Fig. 2, and the mutation frequencies are given in Fig. 1B. These results indicate that T cell–derived signals plus sIg cross-linking are capable of maintaining somatic mutation in vitro. Both of these components seem to be necessary for the continuation of mutation, because coculture of postimmunization day 10 B cells with alloreactive T_H2 cells alone brought about no increase in mutation (Fig. 1B). In view of the pivotal nature of the CD40 molecule in the delivery of T cell help to B cells (11), we also investigated the capacity of a CD40 ligand expressing transfectant to replace T_H2 cells in maintaining somatic mutation in day 10 B cells. These transfectants plus anti-κ–Sepharose did not induce an increase over the day 10 baseline amount (Fig. 1B). Thus, the T_H2 signal that acts to perpetuate mutation activity in cultured B cells seems not to be the CD40 ligand acting alone.

We interpret the increase in mutation after coculture with the T_H2 cells and anti-κ–Sepharose to indicate ongoing somatic mutation in vitro. However, it is possible that these stimuli selectively amplify a minor population of B cells that by day 10 in vivo has a large number of mutations. To differentiate between these two possibilities, we performed limiting dilution culture of B cells from mice immunized with phOx-OVA 10 days earlier. The B cells were plated on 96-well plates with T_H2 cells plus anti-κ–Sepharose or with LPS and were cultured for 8 days (Fig. 3A). From phOx-positive wells (which, according to the Poisson distribution, should be derived from single precursors), we amplified $V_{\kappa Ox1}$-$J_{\kappa 5}$ rearrangements by RT-PCR, cloned these into plasmids, and sequenced them. Figure 3B shows that two clones from LPS-stimulated cultures showed a low frequency of mutation (average 1/830) and no appreciable sequence diversity, whereas four clones from cultures stimulated with T_H2 cells plus anti-κ–Sepharose exhibited a high frequency of mutations (average 1/162) and, more important, extensive intraclonal sequence diversity. Three of the four clones must have arisen from a B cell clone having its $V_{\kappa Ox1}$ gene in germline con-

															CDR3				
58	59	61	63	67	71	74	75	76	78	83	84	85	87	88	89	91	92	93	
V	P	A	S	S	Y	T	I	S	M	A	A	T	Y	C	Q	W	S	S	
GTC	CCT	CGC	AGT	TCT	TAC	ATC	ATC	AGC	ATG	GCT	GCC	ACT	TAC	TGC	CAG	TGG	AGT	AGT	
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---	--C	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	15-164-35
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---	---	---	---	--A	---	---	---	---	---	---	---	---	---	---	---	---	---	---	33-09-22
---	---	---	---	---	---	---	---	---	C--	---	---	---	---	---	---	---	---	---	33-09-33
---	--C	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	33-09-35
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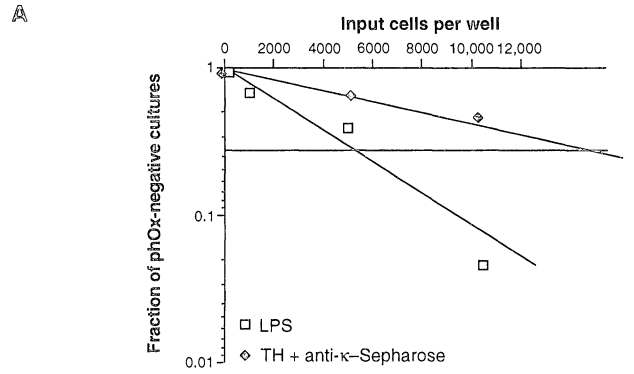
figuration at day 10, whereas one clone (clone 2) had a single mutation at the onset. This result is compatible with our hybridoma data (Fig. 1B), where two of three sequences are in germline configuration at day 10.

We conclude that stimuli delivered by activated T_H2 cells in combination with signals from sIg support somatic hypermutation activity in vitro. The identity of the T cell–derived signal is not known, but it does not seem to be CD40 ligand alone. There is a clear analogy between the culture conditions that support mutation and those that prevail in the site of hypermutation in vivo (5): In germinal centers, Ig cross-linking can occur in the form of antigen-antibody complexes on the surface of FDCs (8), and antigen-specific T cells are present (6, *12*). There are probably insufficient T cells in the germinal center to provide stimuli through interaction involving cell contact, and, given the failure of CD40 ligand to replace the T cell activity in the mutation cultures, a role for soluble mediators in maintaining mutation may be indicated. Until now, the process of somatic mutation has not been divisible into separate phases. It could be argued that the presence of germline sequences at day 10 after priming in our limiting dilution cultures indicates that somatic mutation is being induced in vitro. The failure to induce somatic mutation of B cells primed for 7 days and in naïve B cells (*13*) with the same stimuli argues against such a conclusion. We prefer an interpretation in which the responding B cells have been "committed" to somatic mutation between days 7 and 10 after immunization, and our results provide evidence that B cells that have initiated the mutation process require continued stimulation (by T cells and antigen) to allow mutation to proceed. This "progression" phase can be mimicked in vitro. We conclude that the commitment and the progression of somatic hypermutation are driven by distinct and exclusive signals.



B

	CDR 1						CDR2									CDR3		
	M	S	V	S	K	G	T	K	A	S	G	G	S	T	S	E	A	W
	11	29	30	31	39	41	42	45	55	63	64	68	70	74	76	81	83	91
H3	ATG	AGT	GTA	AGT	AAG	GGC	ACC	AAA	GCT	AGT	GGC	GGG	TCT	ACA	AGA	GAA	GCT	TGG
LPS clone 1 (f: 1/665)																		
1-8	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
1-4	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
1-1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
1-2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
1-6	--C	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
1-9	--C	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
1-7	---	---	---	-A-	---	---	---	---	---	---	---	---	---	---	---	---	---	---
LPS clone 3 (f: 1/998)																		
3-10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-A-	---	---	---
3-5	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
3-6	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
3-2	---	---	---	---	---	---	---	---	--A	---	---	---	---	---	---	---	---	---
3-7	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
3-8	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
3-1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
T_H + anti-κ clone 4 (f: 1/171)																		
4-2	---	---	---	-A-	---	---	---	---	---	---	---	---	---	---	---	-T-	---	---
4-3	---	---	---	---	---	---	---	C--	---	---	---	---	---	---	---	-T-	---	---
4-5	---	---	---	---	---	---	---	C--	---	---	---	---	---	---	---	-T-	---	---
4-6	---	---	---	---	---	---	T--	---	---	---	---	---	--C	---	---	---	---	---
4-8	---	---	---	---	---	---	T--	---	---	---	---	---	---	---	---	---	---	---
4-9	---	---	---	---	---	---	T--	---	---	---	---	---	---	---	---	---	---	---
T_H + anti-κ clone 7 (f: 1/158)																		
7-4	---	---	---	--A	---	---	---	---	---	---	---	---	---	---	---	---	---	---
7-6	---	---	A--	---	---	---	---	---	---	---	-A-	---	---	---	---	---	A--	---
7-9	---	---	---	---	---	---	---	---	---	---	-A-	---	---	---	---	---	A--	---
7-2	---	---	---	---	---	---	---	---	---	---	-A-	---	---	---	---	---	---	---
7-5	---	---	---	---	---	---	---	---	---	---	-A-	---	---	-T-	---	---	---	---
T_H + anti-κ clone 2 (f: 1/130)																		
2-6	---	---	---	---	--C	--A	---	---	---	---	---	---	---	---	---	---	---	---
2-10	---	---	---	---	--C	--A	---	---	---	---	---	---	---	---	---	---	---	-C-
2-8	---	---	---	---	--C	--A	---	---	---	--A	---	---	---	---	---	---	---	---
2-5	---	---	---	---	--C	---	---	---	---	---	---	---	---	---	---	-G-	---	---
2-3	---	---	---	---	--C	---	---	---	---	---	---	---	---	---	---	---	---	---
T_H + anti-κ clone 9 (f: 1/181)																		
9-2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	C--
9-6	---	---	---	G--	---	---	---	---	---	---	---	---	---	---	---	---	---	C--
9-9	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	C--
9-8	---	--A	-C-	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
9-7	---	--A	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
9-11	---	--A	---	---	---	---	---	---	---	---	---	---	---	---	---	-G-	---	---
9-1	---	--A	---	---	---	---	---	---	---	---	---	---	--T	---	---	---	---	---

Fig. 3. (**A**) Frequency of phOx-positive wells versus cellular input for cultures stimulated with LPS and with T_H cells plus anti-κ–Sepharose. (**B**) Sequences from two clones of B cells stimulated by LPS and four clones stimulated by T_H cells (6000 cells per well) plus anti-κ–Sepharose (*18*). Each sequence is shown only once, and the number of independent isolates of each sequence is indicated at the left (f, frequency of mutation). Amino acid abbreviations are as in Fig. 2.

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15. BALB/c mice were immunized intraperitoneally with 100 μg of alum-precipitated phOx coupled to ovalbumin (phOx-OVA), as described (*9*). The spleens were removed aseptically, teased into single-cell suspension, and fused with SP2/0 according to standard procedures or depleted of red cells (with Gey's solution) and T cells [by treatment with anti-theta antibodies and complement (Boehringer)]. Cells were cultured in Iscove's modified Dulbecco's minimum essential medium supplemented with 5% fetal calf serum and antibiotics in the presence of LPS (25 μg/ml) (*Escherichia coli* 055:B5; Difco, Detroit) or 100,000 irradiated (2500 rads) alloreactive T_H cells in the presence or absence of anti-κ [187.1 (*14*)] coupled to Sepharose in 24-well plates. The alloreactive T cell line used was raised as described (*10*) by stimulating BALB/k spleen cells in vitro with irradiated BALB/c stimulators (specificity IA^d) and clones derived by limiting dilution. The in vitro cultures were fused with SP2/0 according to standard procedures at day 4 of culture. Growing hybridomas were screened for phOx specificity by means of enzyme-linked immunosorbent assay (ELISA) with phOx coupled to bovine serum albumin as the coating antigen. First-strand complementary DNA (cDNA) was prepared from NP40 lysates of hybridoma cells with random primers and was amplified with nested $V_{κOx1}$-specific primers [5′ primer (5′-CCGGG-

GAATTCTCAGCTTCCTGCTAATCA-3′) and $J_{\kappa 5}$ primer (5′-ATAGGATCCCGTTTCAGCTCCAGCTTGGT-3′) for 20 cycles, followed by an additional 30 cycles with (5′-GCCGGAATTCCCAGAGGACAAATTGTTC-3′)]. The cDNA PCR products were purified with Magic PCR Preps (Promega) and sequenced with a $J_{\kappa 5}$-specific primer (5′-ACGTTTCAGCTCCAGCTTGGTC-CCA-3′). DNA sequencing was performed with T7 DNA polymerase or with Taq polymerase with the use of di- or dye-deoxy terminators.

16. The source of CD40 ligand was the transfectant cell line K47 (G. Wohlleben *et al.*, *Int. Immunol.*, in press) that constitutively expresses CD40 ligand (CD40L).
17. E. A. Kabat *et al.*, *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1983).
18. T cell–depleted splenic B cells from BALB/c mice immunized with phOx-OVA, as described above (*15*), were stimulated with the indicated stimuli in 96-well plates at the indicated cellular input. After 8 days in culture, the supernatants were screened for phOx reactivity by ELISA, the positive wells were scored, and the cells were harvested. The $V_{\kappa Ox1}$ rearrangements were amplified by RT-PCR as described above and cloned into pGem3Z vector. Random plasmids containing inserts were selected and sequenced. The measured Taq polymerase error under these conditions is less than one mutation per 1000 bases sequenced.
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Role of Lymphotoxin and the Type I TNF Receptor in the Formation of Germinal Centers

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In mice deficient in either lymphotoxin-α (LT-α) or the type I tumor necrosis factor (TNF) receptor, but not the type II TNF receptor, germinal centers failed to develop in peripheral lymphoid organs. Germinal center formation was restored in LT-α–deficient mice by transplantation of normal bone marrow, indicating that the LT-α–expressing cells required to establish this lymphoid structure are derived from bone marrow.

The structurally related cytokines LT and TNF-α can modulate many immune and inflammatory reactions (*1*). LT has been identified in two different molecular forms: a secreted form consisting of an LT-α homotrimer and a membrane-associated heteromeric complex, composed (in its major form) of one LT-α monomer and two identical 33-kD transmembrane LT-β subunits (2–4). The secreted LT-α homotrimer binds and activates both TNF receptor type 1 (TNFR-I) and type 2 (TNFR-II) (*5*), whereas the membrane-associated $LT\text{-}\alpha_1$–$LT\text{-}\beta_2$ heteromer engages a newly identified receptor designated the TNF receptor–related protein (TNFRrp) (*4*, *6*). In vitro, LT duplicates the activities of TNF-α. However, recent studies have demonstrated that LT-α occupies a central, unique role in the development of lymph nodes (LNs) and Peyer's patches (PPs) (*7*). The relative roles of TNFR-I, TNFR-II, and other LT-binding cell surface receptors in transducing LT-specific signals have not been defined (*5*).

Mice rendered deficient in LT-α ($LT\text{-}\alpha^{-/-}$) are born without morphologically detectable LNs or PPs (*7*). The spleen, although present, shows disturbed architecture, with failure in the white pulp of normal segregation of B and T cell zones and failure to establish a distinct marginal zone. These findings suggest that responses that depend on normal cellular interactions within white pulp follicles may be dysfunctional in these mice.

We tested this hypothesis by immunizing normal and $LT\text{-}\alpha^{-/-}$ mice with sheep red blood cells (SRBCs) (*8*). Ten days after immunization, immunohistochemical evaluation of the spleens of wild-type mice demonstrated the formation of prominent germinal centers (GCs), visualized as clusters of cells binding peanut agglutinin (PNA) (*9*) surrounded by cells staining with antibody to immunoglobulin D (IgD) (Fig. 1A). In contrast, spleen sections from $LT\text{-}\alpha^{-/-}$ mice immunized with SRBCs showed no GC structures (Fig. 1B). PNA-binding cells were rarely detected and, when seen, were closely approximated to vascular structures and not associated with IgD-expressing cells. Similar results were obtained after immunization with (4-hydroxy-3-nitrophenyl)acetyl–ovalbumin and trinitrophenyl keyhole limpet hemocyanin. These observations indicate that in addition to supporting the development of LNs and PPs, LT-α has an essential function in supporting the generation of normal GC structure.

After their release in soluble form from cells, both TNF-α and LT-α are homotrimers (*10*). These two cytokines can engage both the 55-kD TNF receptor (TNFR-I) and the 75-kD TNF receptor (TNFR-II) (*3*, *5*). To evaluate whether either of these TNF receptors was required for normal GC formation, we used SRBCs to immunize mice rendered deficient in either TNFR-I ($TNFR\text{-}I^{-/-}$) or TNFR-II ($TNFR\text{-}II^{-/-}$) by gene targeting (*11*). Although $TNFR\text{-}II^{-/-}$ mice had morphologically normal GCs (Fig. 1D), $TNFR\text{-}I^{-/-}$ mice developed no GCs (Fig. 1C), demonstrating that signaling through TNFR-I is also required for GC development. Experiments performed in vitro (*12*) and in vivo (*13*) have suggested unique activities for TNFR-I and TNFR-II. Previous studies of mice rendered deficient in each of these receptors by gene targeting have also iden-

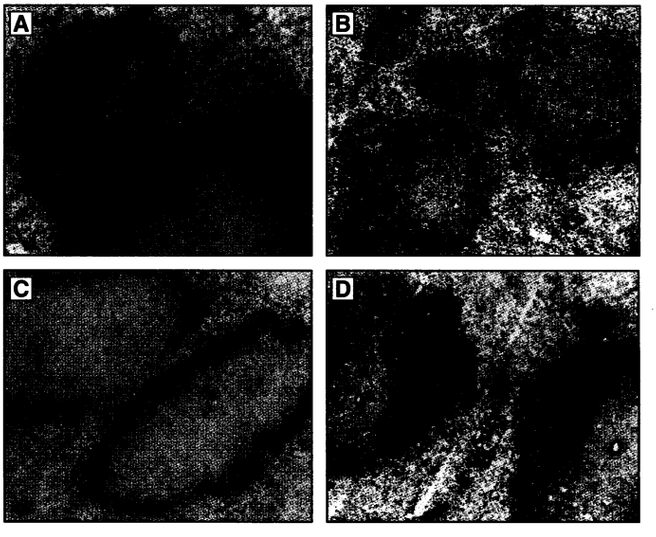

Fig. 1. Absence of GCs in spleens from immunized $LT\text{-}\alpha^{-/-}$ and $TNFR\text{-}I^{-/-}$ mice but not from $TNFR\text{-}II^{-/-}$ mice. Spleen sections from wild-type (**A**), $LT\text{-}\alpha^{-/-}$ (**B**), $TNFR\text{-}I^{-/-}$ (**C**), and $TNFR\text{-}II^{-/-}$ (**D**) mice were stained with PNA (blue) and antibody to IgD (brown). Typical GC development is absent in $LT\text{-}\alpha^{-/-}$ and $TNFR\text{-}I^{-/-}$ mice, but present in wild-type and $TNFR\text{-}II^{-/-}$ mice after immunization with the T lymphocyte–dependent antigen SRBC. Magnification, ×30.

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