Enhanced Fidelity of 3TC-Selected Mutant HIV-1 Reverse Transcriptase

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Monotherapy with (−)2′,3′-dideoxy-3′-thiacytidine (3TC) leads to the appearance of a drug-resistant variant of human immunodeficiency virus–type 1 (HIV-1) with the methionine-184 → valine (M184V) substitution in the reverse transcriptase (RT). Despite resulting drug resistance, treatment for more than 48 weeks is associated with a lower plasma viral burden than that at baseline. Studies to investigate this apparent contradiction revealed the following. (i) Titers of HIV-neutralizing antibodies remained stable in 3TC-treated individuals in contrast to rapid declines in those treated with azidothymidine (AZT). (ii) Unlike wild-type HIV, growth of M184V HIV in cell culture in the presence of d4T, AZT, Nevirapine, Delavirdine, or Saquinavir did not select for variants displaying drug resistance. (iii) There was an increase in fidelity of nucleotide insertion by the M184V mutant compared with wild-type enzyme.

HIV infection is characterized by rapid genetic variation resulting in diverse viral populations in infected individuals (*1*). The generation, over time, of HIV variants that display resistance to drugs directed against viral RT and protease is thought to account, in part, for the ultimate failure of most antiviral strategies (*2*). The rapid appearance of such mutants is a combined result of low-fidelity copying by RT (*3, 4*) and of the selection pressure created by long-term antiviral therapy in HIV-infected individuals. Thus, an effective therapeutic agent might be one that increases the fidelity of HIV RT, thereby diminishing genetic diversification.

The M184V mutation is known to evoke high-level resistance (up to 1000 times as much) to 3TC as well as low-level resistance (3 to 20 times as much) to both 2′,3′-dideoxyinosine (ddI) and 2′,3′-dideoxycytidine (ddC) as compared to the wild type (*5–7*). In clinical studies, resistance to 3TC was observed in nearly all patients who received 3TC monotherapy for more than 12 weeks. The clinical performance of individuals who received 3TC monotherapy has been surprisingly good, considering the high degree of drug resistance observed (*8*). Moreover, the overall viral burden in these patients remained below that at baseline, despite the fact that almost all individuals eventually displayed phenotypic resistance against this compound and possessed the M184V substitution (*7*).

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These observations suggested to us that the emergence of viruses containing the M184V substitution might have been accompanied by a sustained ability of the immune system to suppress viral replication. Median neutralization titers from nine 3TC-treated subjects who were studied over a 48-week period were compared with nine age- and CD4-matched patients who received AZT. The decline in neutralization titers was about seven times as fast in patients receiving AZT (half-life of neutralization titer $t_{1/2}$ = 5.6 weeks) compared with patients who received 3TC ($t_{1/2}$ = 40 weeks) (*9–11*). These data suggested that the M184V substitution may confer increased fidelity to RT, thereby preventing the emergence of HIVs with variant envelope proteins; such variants have been shown to escape neutralization by preexisting antiviral antibodies (*12*) or cytotoxic T lymphocytes (*13*).

To further assess the potential for genetic variability among wild-type and M184V HIV isolates, we used drug-selection protocols (*14*) in which the emergence of drug-resistant HIV variants was monitored over time. The wild-type recombinant HxB2 clone of HIV, the HIV_{IIIB} isolate, and a drug-sensitive clinical isolate could each replicate in the presence of gradually increasing concentrations of AZT, suggesting the appearance of resistant variants (Fig. 1). In contrast, the replication of a recombinant, infectious clone of HIV containing the M184V substitution was inhibited by AZT (*15, 16*). These results were also reproduced with clinical isolates (M184V-clinical-A and -B) that were shown, by mutation-specific polymerase chain reaction, to solely contain the M184V substitution. Replication of M184V-clinical-A (from an individual treated with 3TC) in above-threshold concentrations of AZT was undetectable until 4 to 5 weeks of growth in culture, in contrast with much higher levels of replication seen in 2 to 3 weeks, in the case of virus harvested from

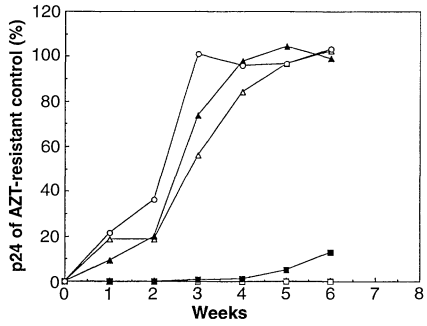

Fig. 1. Selection of resistance to AZT performed with HIV_{IIIB} (△), the HxB2 clone of infectious virus (▲), a drug-naïve clinical isolate (○), a clinical isolate from a patient treated with 3TC for 4 months (M184-clinical-A) (■), and the M184V recombinant clone of HIV (□). Details of selection procedures were described previously (*7*). Measurements of p24 antigen amounts were by the Abbott antigen-capture assay as described (*7*). Results are plotted as percentage of growth observed with the AZT-resistant virus containing the M41L, K70R, and T215Y (Met^{41} → Leu, Lys^{70} → Arg, and Thr^{215} → Tyr) substitutions. Note that, unlike the molecular clone of M184V virus, the clinical isolate containing the M184V substitution appeared to be outgrowing into a resistant viral population. This may reflect the possibility that some residual M184 virus remained in the quasispecies after treatment with 3TC.

Table 1. Time to outgrowth (measured as the appearance of p24) of viruses in the presence of specific drugs.

Virus tested	Time to outgrowth in presence of drug (days)				
	AZT	d4T	Nevirapine	Delavirdine	Saquinavir
HxB2	22	27	15	12	19
HIV_{IIIB}	26	24	14	14	15
HxB2-M184V	>180	>180	>180	>180	>180
M184V-clinical-A	110	97	85	92	135
M184-clinical-B	78	71	62	56	86
HxB2-K65R	24	22	18	14	16

Table 2. The IC_{50}'s of mutant viruses that were compared in the drug selection experiment (summarized in Table 1), where IC_{50} is the amount of a drug required to inhibit viral growth by 50%.

Virus	IC_{50} for drug			
	AZT	d4T	Nevirapine	Delavirdine
HxB2	0.002	0.03	0.3	0.006
HIV_{IIIB}	0.002	0.04	0.2	0.004
HxB2-M184V	0.001	0.03	0.3	0.008
M184V-clinical-A	0.003	0.05	0.5	0.005
M184V-clinical-B	0.001	0.04	0.4	0.004
HxB2-K65R	0.001	0.02	0.6	0.002

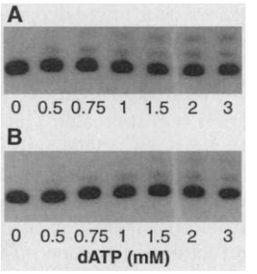

Fig. 2. Autoradiogram showing the effect of dATP (deoxyadenosine triphosphate) concentration on the rate of misinsertion by the wild-type (**A**) and M184V mutant (**B**) RTs. The reactions were run as described in the text, and 6 μl of the reaction was separated on a 16% polyacrylamide-urea gel. The template-primer used was PBS-A (*19*).

drug-naïve patients (Fig. 1). The time required for initial detection of apparent resistance to AZT and other antivirals in tissue culture is summarized (Table 1). For both the drug-sensitive HIV_{IIIB} isolate and recombinant HxB2, periods of 2 to 3 weeks (four to six passages) were usually sufficient for initial indications of viral growth in the presence of above-threshold concentrations of AZT, d4T, Nevirapine, Delavirdine, or Saquinavir. However, during 36 weeks of growth under drug selection, little or no resistance to any of the above compounds was detected with recombinant M184V virus or with clinical isolates containing the M184V substitution. In contrast, K65R ($Lys^{65} \rightarrow Arg$) mutant virus, which is resistant to ddC, could be selected for resistance to these drugs (*17*). The diminished ability to select drug-resistant variants with M184V virus in cell culture virus-replication procedures is consistent with the hypothesis that the M184V mutation confers a greater polymerase fidelity on RT.

One must consider two other explanations for a slowed, or a lack of, outgrowth of drug-resistant variants from M184V virus. The first is a possible decreased rate of replication of the M184V mutant. Growth competition experiments to determine the relative fitness showed that the M184V virus is not significantly compromised in its replicative capacity as compared to the wild type (*15*). Thus, our results showing a lack of virus outgrowth, until 180 days in the presence of drugs (HxB2-M184V in Table 1), cannot be explained by a slight growth disadvantage observed for the M184V virus. Of course, one might expect that the genetic background in which the M184V virus is present, to some degree, could modulate the time required for outgrowth of resistant viruses. In this context, the second clinical isolate with the M184V substitution (M184V-clinical-B in Table 1) grew better than the first in the presence of all of the drugs tested. A second explanation could be that the M184V substitution somehow confers an increased sensitivity to each of the drugs used in our experiments (Table 1). M184V, in combination with the T215Y mutation, is known to cause a reversal of AZT resistance (*5*). However, viruses containing only the M184V substitution did not display heightened sensitivity to any of the anti-RT drugs studied (Table 2). Additionally, it appears improbable that the M184V substitution in RT would influence the sensitivity of protease to Saquinavir. Thus, failure to replicate in the presence of these drugs is not attributable to diminished capacity for replication, diminished fitness, or increased sensitivity to the drugs used in the selection procedure.

To directly address the effect of the M184V alteration on polymerase fidelity, we used a gel-based, steady-state kinetic assay (*18*) to compare the nucleotide misinsertion efficiencies of the wild-type and the M184V variant RTs. A 5′ ^{32}P-labeled DNA primer with partial homology to the cognate primer for HIV RT, transfer RNA $(tRNA)^{lys,3}$, was annealed to a DNA template (45-nucleotide oligomer) representing the sequence around the primer binding site (PBS) of the HIV-1 genome (*19*, *20*). Standing-start polymerization reactions (*21*) were carried out with purified HIV-1 wild-type RT or M184V RT heterodimers (*22*)

Table 3. The V_{max}, the K_m, and the efficiency of misinsertion (f_{ins}) for wild-type (WT) and M184V RTs. Both V_{max} and K_m are relative values. Standard deviations presented are derived from two or three independent measurements; values obtained from three independent measurements are indicated with an asterisk. f_{ins} was evaluated from ratios of relative V_{max} to K_m as described in (*18*). Base pairs are shown with the template first.

Base pair	V_{max} (%/min)		K_m (μM)		f_{ins}	
	WT	M184V	WT	M184V	WT	M184V
AT	3.90 ± 1.00*	2.33 ± 0.10*	3.20 ± 0.55*	2.01 ± 0.25*	1	1
AC	1.05 ± 0.01	0.33 ± 0.09*	811 ± 57.20	850 ± 370*	1.05×10^{-3}	3.93×10^{-4}
AG	0.02 ± 0.00	0.02 ± 0.00*	151 ± 31	260 ± 70*	9.35×10^{-5}	6.79×10^{-5}
AA	1.10 ± 0.02	0.38 ± 0.04	621 ± 144	1210 ± 290	1.52×10^{-3}	2.77×10^{-4}
TA	5.20 ± 0.05	1.32 ± 0.04	0.77 ± 0.11	0.99 ± 0.11	1	1
TG	1.96 ± 0.28	0.15 ± 0.03*	490 ± 70	280 ± 60*	5.72×10^{-4}	3.98×10^{-4}
TC	0.67 ± 0.22	0.02 ± 0.00	1080 ± 220	560 ± 210	8.71×10^{-5}	3.70×10^{-5}
TT	0.63 ± 0.08	0.06 ± 0.01	420 ± 80	2550 ± 150	2.19×10^{-4}	1.73×10^{-5}
GC	2.70 ± 0.21	1.93 ± 0.16	1.16 ± 0.26	1.1† ± 0.3	1	1
GT	6.72 ± 2.70	4.76 ± 0.02*	165 ± 45	570 ± 150*	1.55×10^{-2}	4.73×10^{-3}
GA	0.94 ± 0.09	0.20 ± 0.04*	715 ± 200	500 ± 85*	5.62×10^{-4}	2.18×10^{-4}
GG	0.24 ± 0.05	0.36 ± 0.06	350 ± 60	1400 ± 300	2.97×10^{-4}	1.41×10^{-4}
CG	3.41 ± 0.57	6.33 ± 0.25	0.43 ± 0.12	0.64 ± 0.09	1	1
CA	0.68 ± 0.09	0.17 ± 0.03	330 ± 30	540 ± 10	2.54×10^{-4}	3.07×10^{-5}
CT	0.23 ± 0.04*	0.03 ± 0.01	510 ± 110*	980 ± 110	5.60×10^{-5}	3.20×10^{-6}
CC†	ND	ND	ND	ND	ND	ND

†The misinsertion of deoxycytosine triphosphate opposite template C was not detectable (ND) under the conditions used.

in the presence of a single deoxynucleotide triphosphate (dNTP) (*23*). A typical set of reactions resulted in the increased accumulation of extended primer as the dNTP concentration was increased (Fig. 2). The kinetic constants, K_m (Michaelis constant) and relative V_{max} (maximum velocity) were determined for each set of reactions from the densitometric quantitations of unextended and extended primers (*23, 24*) for every possible insertion event, that is, 4 correct base pairs and 12 mispairs [primers of variable lengths were used to measure misinsertion opposite all four template bases (*19*)]. The relative V_{max} and the K_m values from the 16 insertion events were used to derive f_{ins}, the efficiency of misinsertion, as described (*18, 24*).

The rates of base substitution by HIV-1 RT vary and are influenced by the template base, the dNTP, and the sequence context (*4, 25*). As evident from the f_{ins} values (Tables 3 and 4 and Fig. 3), the M184V mutant displayed a greater fidelity of insertion than did the wild-type HIV-1 RT. A higher fidelity was observed in the formation of every mispair tested, with the enhancement ranging from 2 to 17 times as much for various mispairs. However, decreases in the formation of certain mispairs were greater than those for others (for example, CA, TT, and CT pairs were formed 8 to 17 times less efficiently than the wild type) (Table 4). Our results indicate that the range of misinsertions by the wild-type RT was from 1.55×10^{-2} to 5.6×10^{-5}. The overall increase in fidelity displayed by the M184V RT over that of the wild type (determined by a comparison of the average misinsertion efficiencies) was by a factor of 3.2 and the range of misinsertions by the M184V RT was from 4.73×10^{-3} to 3.2×10^{-6}.

The magnitude of the increase in fidelity revealed by our studies of misinsertion could be a minimal estimate of that in vivo. For example, misextension, the extension of a mispaired primer caused by misinsertion, can help to "fix" a misinsertion (*26*). Similarly, errors mediated by template or primer slippage lead to base substitutions, deletions, and insertions (*27*). The influence of M184V alteration on either of these types of errors is not known. Further investigations that include experiments in a single infectious cycle with reporter HIV constructs are needed to address this issue. Considering the fact that the rate of genetic variation in HIV is at the threshold for survival, any slowing of genetic variation is likely to influence host-virus interactions (*28*).

It has been argued that the effect of increased fidelity on viral evolution may be irrelevant considering the high rates of replication observed for HIV-1 (*29, 30*). Although it is likely that a marginal increase in fidelity might result in a short time period during which the virus remains at low titers, the emergence of other mutations (alone or in combination with M184V) could lead to higher increases in fidelity and hence a greater window of suppressed viral titers.

An additional benefit of a slower rate of evolution of the HIV-1 quasispecies is the effect on the ability of the immune system to eliminate viruses that would be less proficient in generating immunological escape mutants. A continued effectiveness of anti-HIV immune responsiveness in treated individuals is suggested by the observed drops in viral burden with high-level phenotypic resistance to 3TC and the protracted capacity of sera to neutralize viral isolates obtained at multiple sequential times. If our hypothesis of continued immune effectiveness is correct, why did the viral burden not continue to fall indefinitely in 3TC-treated individuals? The answer may lie in the fact that the high mutation rate of HIV-1 RT (10^{-4} to 10^{-5}) leads to a high proportion of defective virus particles (*31*). However, in the case of a M184V RT with enhanced fidelity, a much higher proportion of virus replicative events would be expected to yield infectious progeny (*32*).

A variant RT with increased fidelity may have implications for management of HIV disease. The real value of 3TC may be as a means of selecting for the M184V substitution rather than as an antiviral compound with the potential to impede viral replication over long periods. Subsequent to the initiation of 3TC therapy, a quantitative picture of the proportion of M184V virus in the HIV quasispecies could be obtained before administering a second drug directed against a different viral component such as protease. Thus, the virus population would not only be sensitive to the antiprotease drug but might be less likely to develop resistance to it.

Note added in proof: Recent results with misinsertion assays have revealed that another dideoxynucleoside analog–resistant RT, the E89G variant, polymerizes with an increased dNTP insertion fidelity (*37*).

Table 4. Fold increases in the misinsertion fidelity of M184V RT. The decreases in the efficiency of misinsertions of the mutant RT over those of the wild type were calculated from the data in Table 3. ND, not determined.

Mispair	Fold increases for M184V
AC	2.67
AG	1.37
AA	5.48
TG	1.44
TC	2.35
TT	12.65
GT	3.28
GA	2.58
GG	2.11
CA	8.27
CT	16.97
CC	ND

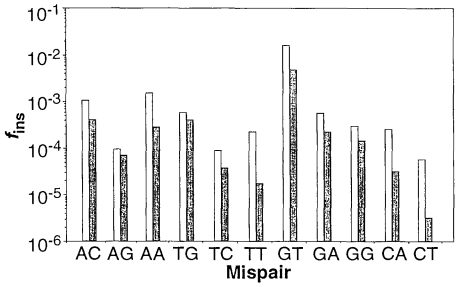

Fig. 3. Misinsertion efficiencies of wild-type (□) and M184V (■) RTs. A comparative plot of misinsertion efficiencies (f_{ins}) experimentally determined for 11 of the 12 possible mispairs for wild-type and M184V RTs (from Table 3) is shown.

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15. The M184V substitution does not adversely affect viral replication (*33*). In fact, M184V-containing viruses replicate and kill T cells as rapidly as do wild-type viruses (*33*). Furthermore, clinically derived viruses with M184V were pathogenic for both primary lymphocytes and established cell lines and possessed the syncytium-inducing phenotype (*34*).
16. No RT mutations associated with AZT resistance at positions 41 and 215 were detected in these samples, whereas these mutations were present in wild-type viruses after selection for resistance.

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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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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