Incorporation of dUTP does not mediate mutation of A:T base pairs in Ig genes in vivo

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ABSTRACT

Activation-induced cytidine deaminase (AID) protein initiates Ig gene mutation by deaminating cytosines, converting them into uracils. Excision of AID-induced uracils by uracil-N-glycosylase is responsible for most transversion mutations at G:C base pairs. On the other hand, processing of AID-induced G:U mismatches by mismatch repair factors is responsible for most mutation at Ig A:T base pairs. Why mismatch processing should be error prone is unknown. One theory proposes that long patch excision in G1-phase leads to dUTP-incorporation opposite adenines as a result of the higher G1-phase ratio of nuclear dUTP to dTTP. Subsequent base excision at the A:U base pairs produced could then create non-instructional templates leading to permanent mutations at A:T base pairs (1). This compelling theory has remained untested. We have developed a method to rapidly modify DNA repair pathways in mutating mouse B cells in vivo by transducing Ig knock-in splenic mouse B cells with GFP-tagged retroviruses, then adoptively transferring GFP+ cells, along with appropriate antigen, into primed congenic hosts. We have used this method to show that dUTP-incorporation is unlikely to be the cause of AID-induced mutation of A:T base pairs, and instead propose that A:T mutations might arise as an indirect consequence of nucleotide paucity during AID-induced DNA repair.

INTRODUCTION

Somatic hypermutation (SHM) of antibody (Ig) genes in antigen-activated germinal centre B cells optimizes the antibody repertoire to maintain effective immunity (2). SHM is initiated by deamination of cytosine (C) bases in Ig V genes by activation-induced cytidine deaminase (AID), which produces mismatched uracil:guanine (U:G) base pairs [reviewed in (3)]. If left unprocessed, U:G base pairs are inherited as a thymine:adenine (T:A) base pair (i.e. a C:G to T:A transition mutation) in one daughter cell following replication (4), but excision of AID-induced uracils by uracil N-glycosylase (UNG) creates a non-instructional template on one strand. Such templates can induce bypass by low fidelity translesion DNA polymerases, potentially converting the founder C base into any other base in one daughter cell [i.e. producing transversion or transition mutations at C:G base pairs (5)]. Alternatively, processing of founder U:G mismatches by the mismatch repair heterodimer MutSα (or, to a minor extent, by UNG) introduces mutations at nearby A:T base pairs via recruitment of the translesion DNA polymerase (pol) η (6). Non-homologous end-joining factors are also recruited in response to AID-induced DNA damage, both to mediate class switch recombination and to inhibit homologous recombination or translocation (7–10), but there is no evidence for their direct involvement in generating point mutations (11).

How mismatch processing of AID-induced U:G base pairs occurs and why repair occurs with low fidelity is unknown. While it is clear that mutation of Ig A:T base pairs occurs and why repair occurs with low fidelity is unknown. While it is clear that mutation of Ig A:T base pairs is largely dependent on MutSβ (MSH2 plus MSH6 proteins), the role of MutLα in SHM is more controversial [reviewed in (3)], even though this factor is vital for ‘classical’ post-replication mismatch repair [reviewed in (12)]. In addition, the mechanism that recruits pol η during repair of AID-induced U:G mismatches is unknown. The MutSβ sub-unit MSH2 can bind and activate pol η (13). However, much of the MSH2-mediated repair of non-Ig genes targeted by AID as ‘bystander genes’ is error-free (14), implying that MSH2 is not obliged to recruit pol η when processing AID-induced U:G mismatches. Furthermore, translesion polymerases may usually be activated by mono-ubiquitinated PCNA to
promote DNA synthesis past non-instructional templates, such as abasic sites (15). AID-induced A:T mutation involves PCNA mono-ubiquitination (16), but mismatch processing of U:G base pairs would not be expected to produce a non-instructional template. Neuberger et al. (1) proposed that incorporation of dUTP, in place of dTTP, during processing of mismatches in cell-cycle phase G1 might explain why pol η is recruited during SHM. Nuclear dUTP levels are presumed to be elevated during G1-phase as a result of reduced accumulation of mRNA coding for nuclear dUTPase (17–19), implying that any unscheduled DNA synthesis that occurs in G1-phase cells will involve some incorporation of dUTP in place of dTTP opposite adenine bases. Processing of AID-induced U:G mismatches by MutSξ in G1-phase could therefore generate U:A base pairs during excision patch re-synthesis. Subsequent base excision at U:A base pairs would then create abasic sites opposite A (rather than G) requiring the recruitment of a translesion DNA polymerase—i.e. pol η—for replication (1) (Figure 1). The ‘dUTP-incorporation’ hypothesis potentially explains why mismatch repair of AID-induced U:G mismatches appears to introduce mutations almost exclusively at A:T base pairs, because it proposes preferential use of pol η to bypass abasic sites generated at A:T base pairs (Figure 1).

The dUTP-incorporation hypothesis infers that the maintenance of nuclear dUTPase activity throughout the cell cycle should suppress AID-induced mutation of A:T base pairs. Because models of AID-induced A:T mutation can currently be tested in vivo only, we developed a system to perform rapid transgenesis of B cells hypermutating in vivo to introduce mutations almost exclusively at A:T base pairs (1). The dUTP-incorporation hypothesis infers that the maintenance of nuclear dUTPase activity throughout the cell cycle should suppress AID-induced mutation of A:T base pairs. Because models of AID-induced A:T mutation can currently be tested in vivo only, we developed a system to perform rapid transgenesis of B cells hypermutating in vivo and used it to show that constitutive expression of mouse or EBV dUTPase in the nucleus of mutating B cells does not reduce mutation of A:T base pairs. Surprisingly, constitutive expression of mouse dUTPase significantly increased mutation at A:T base pairs, by a mechanism that appeared to involve the MSH2 protein. We propose that error-prone pol η may be recruited to Ig genes because AID induces Ig mismatch repair when nuclear dNTP levels are inadequate to support processive DNA synthesis by conventional DNA polymerases.

MATERIALS AND METHODS

Mice

*SWHEL* and *SWHELtag1−/−* mice (20,21) were bred on C57BL/6J backgrounds under specific pathogen free conditions in the animal care facility of the Centenary Institute. *Msh2−/−* mice (22) were back-crossed >10 times onto C57BL/6J mice and crossed with *SWHELtag1−/−* mice. C57BL/6J host mice were purchased from Animal Resources Centre (Canning Vale, Western Australia). All mice were used in accordance with approvals issued by the University of Sydney Animal Ethics Committee.

Reagents

PCR amplifications for making constructs used Phusion DNA polymerase (Finnzymes, Finland). Other enzymes used to digest and ligate DNA were purchased from New England Biolabs (Ipswich, MA, USA). Recombinant EBV dUTPase was purified as described earlier (23). Culture supernatants containing soluble mouse CD40L were produced by transiently transfecting HEK293 cells (using Lipofectamine 2000, Invitrogen, Carlsbad, CA, USA) with a plasmid that expresses truncated mouse CD40L fused to the FLAG-epitope (24). All other chemicals, oligonucleotides or purified proteins were purchased from Sigma-Aldrich (Sydney, Australia), unless otherwise stated.

DNA constructs

Human *DUT-N* cDNA was amplified from Ramos cells (ATCC number CRL-1923) using oligonucleotide primers 439 (GCC GGA TCC GGC CGC CAT GCC CTC GCT TGA AGA GAC—restriction site used for cloning underlined) and 440 (TTG CTC GAG CTA TTA ATT CTT TCC AGT GGA ACC AAA ACC). Mouse *DUT-N* cDNA was amplified from A20 cells (ATCC number TIB-208) using primers 469 (TCC GAA TTC CAT TCT TCC GAG GAG ATC ACC CC, deleting the stop codon) or 468 (CCA GGG CCC CAT TCT TCC CGG TGG AGC CGA AGC, deleting the stop codon). Recombinant EBV dUTPase was purified as described earlier (23). Culture supernatants containing soluble mouse CD40L were produced by transiently transfecting HEK293 cells (using Lipofectamine 2000, Invitrogen, Carlsbad, CA, USA) with a plasmid that expresses truncated mouse CD40L fused to the FLAG-epitope (24). All other chemicals, oligonucleotides or purified proteins were purchased from Sigma-Aldrich (Sydney, Australia), unless otherwise stated.

Figure 1. The deoxyuridylate-incorporation model for AID-induced mutation of A:T base pairs as proposed by Neuberger et al. (1).
GCC AAA GAT TGT CGG) to create R171A and S172A mutations (underlined), and primers 494 and 495 (CAT GTT GGC ATC ATC AAT CCC GGC TAC ACG GGG and CCC CGT GTA GCC GGG ATT GAT GAT GCC AAC ATG) to create a D76N mutation (underlined). Coding sequences with stop codons were directly cloned into the pMIG vector (25) for co-expression with GFP via an internal ribosome entry site (IRES), or cloned minus the stop codon into vector pEGFP-N1 (Clontech) to fuse with the N-terminal methionine of EGFP via the vector-encoded linker peptide ‘GARDPPVAT’. DUT-GFP fusion sequences were then sub-cloned into the pMIG vector to replace the IRES and GFP sequences (Figure 3A). The sequences of all constructs assembled using PCR were checked by automated fluorescent sequencing (AGRФ, Brisbane).

### Nuclear protein extraction and western blotting

Transduced GFP⁺ NIH/3T3 or A20 cells (1–10×10⁶) were resuspended in 0.4 ml ice-cold buffer A [10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 100 µg/ml AEBSF, 1 µg/ml Aprotinin and 1 µg/ml Pepstatin (Roche Diagnostics)]. Following incubation on ice for 15 min to swell the cells, 25 µl of ice-cold 10% (v/v) NP-40 was added and the cells were pipetted briefly to disrupt plasma membranes. The lysed cell samples were centrifuged at 12000g at 4°C for 30 min. Pelleted nuclei were resuspended in 100 µl of ice-cold buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 100 µg/ml AEBSF, 1 µg/ml Aprotinin and 1 µg/ml Pepstatin), pipetted for 2 min, and incubated on ice for 30 min with occasional pipetting. Samples were centrifuged at 12000g for 5 min at 4°C and the supernatant (the ‘nuclear protein’ fraction) was transferred to new tubes. All samples were stored at −70°C. Approximately 20 µg aliquots of protein were electrophoresed on 4–12% NuPAGE Bis–Tris (± MES buffer) polyacrylamide gels (Invitrogen) and transferred to PVDF membranes using an iBlot apparatus (Invitrogen). PVDF membranes blocked with 5% (w/v) skim milk powder dissolved in 10 mM Tris–HCl, pH 7.4, 0.9% (w/v) NaCl and 0.1% (v/v) Tween-20 (TBST) were electrophoresed on 4–12% NuPAGE Bis–Tris (± MES buffer) polyacrylamide gels (Invitrogen) and transferred to PVDF membranes using an iBlot apparatus (Invitrogen). PVDF membranes blocked with 5% (w/v) skim milk powder dissolved in 10 mM Tris–HCl, pH 7.4, 0.9% (w/v) NaCl and 0.1% (v/v) Tween-20 (TBST) were incubated overnight at 4°C with rabbit anti-GFP, antiserum (Abcam, Cambridge, UK) or rabbit anti-human nuclear dUTPase (Imgenex, San Diego, CA, USA) diluted in TBST containing 5% (w/v) BSA. Washed membranes were then incubated with goat anti-rabbit IgG1 conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. Bound antibodies were revealed on washed and blotted membranes by incubation with Immobilon chemiluminescent HRP substrate (Millipore) and luminescence was recorded with a Kodak Image Station 4000MM Digital Imaging System (Kodak, NY, USA).

### Retroviral transduction and adoptive transfer

Near-confluent Phoenix-Eco cells (75 cm², Orbigen, San Diego) were transiently transduced using Lipofectamine 2000 (Invitrogen) with 15 µg pMIG plasmid. Following culture for 48 h at 32°C in B cell medium [IMDM supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids, 10 mM HEPES (pH 7.4), 100 µg/ml streptomycin, 100 U/ml penicillin, and 5×10⁻⁵ M 2-mercaptoethanol], culture supernatants were filtered and stored at −70°C. Fresh SWHELag1⁻/⁻ splenocytes were separated from red blood cells using Histopaque 1083, then activated at 37°C in six-well plates at a density of 10⁶ cells/ml with soluble recombinant CD40L-FLAG fusion protein and anti-FLAG antibody (4 µg/ml clone M2) for 1 day in B cell medium. Splenocytes were ‘spinoculated’ (1100g, 45 min, 20°C) with 2×-diluted retrovirus supernatants in the presence of 8 µg/ml Polybrene, washed, then incubated for a further 2 days in activating medium. A second spinoculation was performed the day after the first spinoculation, if necessary. GFP⁺ cells (usually 5–30% of live cells) were purified using a FACS-Vantage DIVA cell sorter (BD Biosciences). Sorted cells were mixed with HEL-SRBC [prepared as described earlier (26)] and injected via a tail vein into 8- to 12-week-old hosts which had been primed i.p. with 10⁸ SRBC 5–7 days earlier. Each host received 1×10⁴ to 5×10⁴ GFP⁺ cells, along with 10⁸ HEL-SRBC in a bolus of 0.25–0.5 ml B cell medium.

### Cell-cycle analysis

Transduced cells were incubated at 10⁷ cells/ml in complete medium plus 10 µg/ml Hoechst 33342 at 37°C for 30 min. Washed cells were resuspended in complete medium and kept ice-cold for FACS analysis.

### Collection of mutation data

Six or seven days after adoptive transfer, single viable GFP⁺, HEL-binding cells were FACS-sorted, using single cell mask settings, into individual wells of 96-well PCR plates (4titude, UK) containing 15 µl/well of ice-cold Mg-free 1× Taq DNA polymerase buffer (Promega ‘Go-Taq’ buffer) supplemented to include 0.1 mM EDTA, 1% Tween-20 detergent and 0.25 mg/ml proteinase K (Roche Diagnostics). Zero cells were sorted into three or more non-adjacent wells to provide negative controls. Plates were sealed, snap frozen and stored at −70°C. Thawed plates were briefly centrifuged, incubated at 56°C for 40 min to digest protein, then at 95°C for 5 min to inactivate proteinase k. Amplification of the single HyHEL10 VDJH gene rearrangement present in each well was performed by nested PCR as follows. Digested cells were diluted to a volume of 25 µl consisting of 1× PCR buffer, 2 mM MgCl₂, 0.2 mM dNTP, 0.5 U recombinant Taq DNA polymerase (Invitrogen), and 1.0 pmol each of primers 85 (TTG AAG CTT AAA AGA TGA ACC GAG GCT AGA TGCC) and 802 (CAG AAT TCT GAG ACC GAG GCT AGA TGCC) and consisted of 40 cycles of 95°C for 15s, 55°C for 1 min and 72°C for 1 min to produce a 1.07 kb product. Secondary PCR reactions (27.5 µl primary PCR aliquots as templates,10 pmol each of primers 452 (TTG AAG CTT AAA AGA TGA ACC GAG GCT AGA TGCC) and 503 (CAG AAT TCT GAG ACC GAG GCT AGA TGCC) and consisted of 40 cycles of 95°C for 15s, 60°C for 30s and 72°C for 1 min to give a
Table 1. Summary of mutations detected in adoptive SWHEL B cells recovered after adoptive transfer

| Transduction with | Cell genotype | No. of Mice | No. of Sequ | Mut’d Seqs | Mutations | No. of point mutations at: | GC:AT ratio | P-valueb | Freq (%) mutateda | G:C | A:T |
|-------------------|--------------|-------------|-------------|------------|-----------|--------------------------|-------------|---------|-----------------|------|------|
| 1 ires GFP        | SWHEL        | 5           | 125         | 77         | 165       | 39 17 32 6 58 13 1.3     | 0.19        | 0.32    | 0.11            |
| 2 ugi ires GFP    | SWHEL        | 1           | 67          | 36         | 83        | 28 0 19 0 29 7 1.3 ns    | 0.00        | 0.35    | 0.12            |
| 3 mDUT ires GFP   | SWHEL        | 3           | 117         | 88         | 304       | 46 25 38 12 120 54 0.9 *  | 0.25        | 0.73    | 0.39            |
| 4 vDUT ires GFP   | SWHEL        | 6           | 191         | 92         | 187       | 38 13 29 6 85 16 0.9 *   | 0.00        | 0.39    | 0.11            |
| 5 vDUT-3X ires GFP| SWHEL        | 2           | 99          | 56         | 117       | 15 8 16 6 49 23 0.6 **   | 0.13        | 0.45    | 0.26            |
| 6 ires GFP        | swHEL        | 3           | 152         | 80         | 141       | 38 18 74 4 6 1 19.1      | 0.15        | 0.00    | 0.00            |
| 7 mDUT ires GFP   | swHEL        | 3           | 144         | 80         | 172       | 48 22 95 4 2 1 56.3 ns   | 0.02        | 0.40    | 0.14            |
| 8 vDUT ires GFP   | swHEL        | 3           | 192         | 79         | 147       | 56 10 69 2 7 3 15.3 ns   | 0.00        | 0.00    | 0.00            |
| 9 vDUT-3X ires GFP| swHEL        | 2           | 137         | 57         | 108       | 35 6 61 3 1 1 36.0 ns    | 0.00        | 0.00    | 0.00            |
| 10 PCR error control | SWHEL      | –           | 188         | 2          | 2         | 1 0 1 0 0 0               |             |         |                 |
| 11 No prime control | SWHEL      | 2           | 63          | 6          | 9         | 2 0 3 0 3 1               |             |         |                 |

Data were pooled from the number of mice indicated.

*Frequency of G:C or A:T base pairs that have undergone mutation, expressed as a percentage.

P-values from contingency tables (Fisher’s exact test) for numbers of mutations at G:C and A:T base pairs, compared to cells of the same genotype transduced with the control ires-GFP construct. **P<0.01; *P<0.05; ns, not significant.

Mutations detected in sequences amplified from single SWHEL B220–CD4+ (i.e. non-B cell) blood cells.

Mutations detected in transduced cells 7 days after adoptive transfer into a host that had not been primed with SRBC previously.

Ts: transition mutation, Tv: transversion mutation.

1.02-kb product. DNA from successful amplifications (generally 65–80% of wells) was treated with exonuclease 1 and alkaline phosphatase (27), then 523 bp of sequence was determined by Macrogen Inc (Seoul, Korea) or the Australian Genome Research Facility (Brisbane, Australia) using primer 453 (CAA GGA TCC CAA CTT CTC TCA GCC GGC).

Analysis of mutations

All mutations were manually confirmed using Sequencher software (Gene Codes Corp.). No mutation was counted if two or more bases were detected at one position, to exclude bases incorporated erroneously by Taq DNA polymerase during PCR. Sequences were sorted according to the phylogenetic tree generated for each mouse using MacVector software (MacVector, Inc.). Custom Excel spreadsheets (Microsoft Corporation) automatically collated mutations. Prism software (GraphPad Software Inc.) was used for all statistical analyses. Fisher’s exact tests were used to detect significant variation in the G:C to A:T mutation ratio, as shown in Table 1. Non-parametric (Kruskal–Wallis) ANOVA, including Dunn’s multiple comparison tests, was used to identify significant changes in mutation frequencies shown in Figure 5.

Measurement of dUTPase function

Nuclear extracts from transduced NIH/3T3 cells were incubated with rabbit anti-GFP antiserum (0.6 μl per 44 cm² cell culture) in buffer B for 0.5 h on ice, then incubated with paramagnetic Dynabeads conjugated to goat anti-rabbit IgG (Invitrogen) for 1 h at 4°C. Washed beads were resuspended in buffer B and samples subjected to semi-quantitative anti-GFP western blotting, using serially-diluted recombinant GFP (a gift from Kimberley Vincent and Mikka Jormakka, Centenary Institute) as a standard. Bead concentrations were then adjusted to normalize GFP concentrations. Normalized bead aliquots were incubated for 1 h with 100 pmol dUTP or dTTP at 37°C in 20 μl buffer NEB2 (New England Biolabs). dUTP or dTTP remaining after incubation was measured using a modified primer extension assay (28). Briefly, primer oligonucleotide 379 (biotin-TGG GAG AAG TTA GTG AGT CCT AAC TTC TCC CA-3) was annealed, then 3.2 pmol aliquots incubated in triplicate reactions in 96-well PCR plates at 42°C for 45 min with 0.5 units exonuclease-free Klenow DNA polymerase fragment, 29 pmol 3H dATP (GE Healthcare) and 1.6 μl extract-treated dUTP or dTTP (equivalent to 8 pmol dUTP or dTTP prior to dephosphorylation), in a 50 μl reaction containing 1× buffer NEB2 and 0.1 mg/ml BSA. Reactions to generate standard curves were identical, but used dUTP or dTTP standards (0–10 pmol). Completed reactions were quantitatively transferred to 96-well streptavidin-coated ScintiPlates (Perkin Elmer) and incubated for an additional 30 min at room temperature to allow streptavidin-mediated recruitment of incorporated 3H into proximity with the solid phase scintillant. Tritium incorporation was determined by scintillation counting in a Perkin Elmer ‘Wallac Microbeta Trilux’ model 1450 liquid scintillation counter.

RESULTS

Rationale: retroviral gene expression in adoptive germinal centre B cells

To test the dUTP-incorporation hypothesis, we aimed to express dUTPase throughout the cell cycle in germinal centre B cells. To this end, we exploited an Ig double-transgenic (SWHEL) mouse line first described by...
Phan et al. (20). SWHEL mice are derived from a C57Bl/6 embryonic stem cell in which the VDJH-rearrangement from the HyHEL10 hybridoma was targeted to the IgH locus. SWHEL mice also carry the HyHEL10 VJ/C20-rearrangement as a randomly-integrated low copy number transgene. Together, the IgH and Igk transgenes confer specificity for the antigen hen egg lysozyme (HEL) upon ~5–10% of the B cells present in the circulation. The elimination of receptor editing capacity by crossing into a RAG-deficient background confers HEL-specificity on 100% of SWHELrag1 B cells (21). A tk-neo cassette was present upstream of the transgenic VDJH exon in the original SWHEL mouse strain (20). Because this cassette may interfere with SHM of the nearby VDJH-region (data not shown), it was deleted by cre-mediated recombination to produce the SWHEL[tk-neo] sub-line used here (26). For simplicities sake, we shall refer to the SWHEL[tk-neo] sub-line as SWHEL in this report.

We activated SWHEL[tk-neo] splenocytes in vitro with recombinant CD40L (Tnfsf5 or CD154), then retrovirally-transduced activated cells with pMIG vectors (25) (Figure 2A–B). The frequency of transduced (i.e. GFP+) cells ranged between 1 and 30%, implying that nearly all GFP+ cells carried only one retroviral integrant. Transductions were carried out over 3 days of culture prior to adoptive transfer, to allow double transduction to be performed if required. After 3 days culture, GFP+ cells were purified by FACS and 10^4 transduced cells transferred to congenic hosts, along with HEL-SRBC as antigen, by tail vein injection (Figure 2B and C). Transduced cells proliferated in adoptive hosts (Figure 2D), but only if those hosts had been primed with SRBC at least 5 days earlier (Supplementary Figure S1). Proliferation was antigen specific, because cells adoptively transferred with mock-conjugated SRBC, instead of HEL-conjugated SRBC, did not proliferate, even in primed hosts (Supplementary Figure S1). Several days after adoptive transfer, transduced SWHEL B cells (i.e. HEL-binding, GFP+ cells) were FACS-sorted from host spleens at one cell per well into 96-well PCR plates (Figure 2D). Nested PCR amplification of the single HyHEL10 VDJH-allele present in each well, followed by direct automated sequencing of PCR products (Figure 2E and F), showed that transduced cells underwent mutation quantitatively comparable to ‘typical’ SHM [‘typical’ SHM is summarized in (3)]. That is, mutation frequencies were A>G>C>T (referring to the non-template strand, Table 1, row 1); G:C transversions were favoured over G:C transitions, especially when C was in the non-coding strand (Table 1, row 1); and hotspots occurred at WRC motifs (data not shown).

During single-cell PCR, Taq DNA polymerase can incorporate incorrect bases. If this happens early in the PCR reaction, it results in a detectable ‘heteroduplex’ mutation: a site where more than one base is detected in a single read. As long as heteroduplex mutations were justifiably ignored, the contribution of PCR error to mutations scored was negligible (Table 1, row 10). Furthermore, mutation prior to transduction was also negligible, because transduced cells transferred to non-primed hosts accumulated very few mutations (Table 1, row 11).
The utility of our transduction/adoptive transfer model was verified using the uracil glycosylase inhibitor protein (ugi) from bacteriophage. In accordance with seminal work with ung/C0 mice and ugi-transfected DT40 cells (5,29), retroviral expression of ugi protein significantly suppressed transversion mutation at G:C base pairs in adoptive SWHEL cells, while having little effect on mutation at A:T base pairs (Table 1 row 2).

Testing the dUTP-incorporation hypothesis

The dUTP-incorporation hypothesis requires the presence of a pool of dUTP in the nucleus of cells undergoing AID-induced mismatch repair (1). To reduce this pool, we cloned mouse and human nuclear dUTPase cDNAs (herein called mDUT or hDUT, respectively) into pMIG retroviral vectors (25). Constructs were engineered to express nuclear dUTPase as either a polypeptide fused to the N-terminus of GFP or as a polypeptide distinct from GFP via an internal ribosome entry site (IRES) (Figure 3A). Because viral proteins are less likely to be subject to post-translational mechanisms which might regulate mouse or human dUTPase activity, we also cloned the EBV dUTPase gene (herein called vDUT) from the EBV-infected Raji cell line (Supplementary Figure S2).

Mouse A20 B cells and NIH/3T3 fibroblasts were transduced to express GFP ± hDUT, mDUT or vDUT. Western blotting of asynchronous cells (i.e. representing all cell-cycle phases) confirmed ectopic DUT expression in transduced cells and also confirmed that GFP was detectable only as a fusion protein when cells were transduced with DUT-GFP fusion constructs (Figure 3B). hDUT expressed poorly in the mouse cells and was not used in further experiments. Green fluorescence was highly concentrated in the nucleus of the majority of NIH/3T3 cells transduced to express mDUT-GFP or vDUT-GFP fusion proteins, whereas green fluorescence was much more disseminated in cells transduced to express GFP alone (Figure 3C). Accumulation of DUT-GFP fusion proteins appeared to be ~150% higher in S/G2 compared to G1 phase cells (Figure 3D), but this probably reflects the larger size of cells with ≥2n DNA content (Supplementary Figure S3A). mDUT and vDUT migrated as single antibody-reactive bands (Figure 3B). Thus, we found no evidence for differential accumulation or obvious post-translational modification of mDUT or vDUT during the cell cycle, although phosphorylation of Ser11 in hDUT or mDUT does not cause a detectable change in SDS–PAGE mobility (36).

To assess the catalytic activity of DUT constructs, DUT-GFP fusion proteins were enriched from nuclear extracts of transduced NIH/3T3 cells using anti-GFP coupled to magnetic beads, then the ability of bead-bound DUT-GFP fusion proteins to degrade dUTP or dTTP was assessed (‘Materials and Methods’ section). Both vDUT-GFP and mDUT-GFP fusion proteins degraded dUTP in preference to dTTP (Figure 4). To confirm that we were detecting genuine dUTPase activity, we made a vDUT mutant: vDUT3X constructs expressed vDUT protein in which three amino acids known to interact

Figure 3. Expression of recombinant DUT proteins in transduced cells. (A) Retroviral constructs used to express DUT as fusion proteins with GFP, or co-translated with GFP via an IRES. (B) Blots of nuclear proteins from transduced mouse A20 B cells (lanes 1–6) or transduced NIH/3T3 cells (lanes 7–9), probed with (top) anti-hDUT antibody cross-reactive with mDUT but not vDUT, or (bottom) anti-GFP antibodies. Black arrows indicate DUT proteins, white arrows indicate GFP, and black/white arrows indicate DUT-GFP fusion proteins. Predicted molecular masses of polypeptides—ignoring post-translational modifications—are: GFP, 27 kDa; hDUT, 18 kDa; mDUT, 17 kDa; vDUT-GFP, 59 kDa; mDUT3X-GFP, 59 kDa. (C) Localization of green fluorescence in NIH/3T3 cells stably transduced to express GFP or DUT-GFP fusion proteins. Images i–iii are from confocal microscopy, while image iv is from conventional light microscopy. (D) Histograms generated from FACS analysis of NIH/3T3 cultures transduced to express (left) mDUT-GFP fusion protein, or (right) vDUT-GFP fusion protein. The histograms depict the following: (Top) DNA content of (grey histograms) GFP+ cells, or (hollow black histograms) 2n DNA content. (Bottom) GFP fluorescence in GFP+ cells with (grey histograms) >2n DNA content, or (hollow black histograms) ≤2n DNA content.
with substrate—Asp76, Arg171 and Ser172 [see (23)]—were changed to Asn, Ala and Ala, respectively (Supplementary Figure S2). The 3X mutations did not appear to substantially affect protein folding because accumulation of vDUT3X-GFP fusion protein in the nucleus was indistinguishable from accumulation of vDUT-GFP fusion protein (Figure 3). As expected, vDUT3X-GFP fusion protein possessed no detectable dUTPase activity (Figure 4). We also FACS-purified cells expressing vDUT-GFP in G1- or S/G2-phases (Supplementary Figure S3). Semi-quantitative blotting confirmed that slightly more vDUT-GFP was extractable from S/G2-phase nuclei, but the dUTPase activity of G1-phase vDUT-GFP and S/G2-phase vDUT-GFP were very similar (Figure 4E).

Constitutive dUTPase expression does not reduce mutation at A:T base pairs

Expression of vDUT in activated B cells in vivo appeared to be somewhat cytotoxic and/or anti-proliferative because vDUT-transduced cells were more difficult to recover after 7 days in adoptive hosts than cells transduced with GFP or mDUT plus GFP (data not shown), and their level of mutation was lower than control cells (Table 1). Among other explanations, vDUT expression may have induced selection for cells that had undergone less proliferation in vivo. The ability of vDUT to activate toll-like receptors (30) might be related to this observation. Because SHM depends on proliferation, and also because we were interested in relative mutation of G:C versus A:T base pairs, statistical analyses in Table 1 and Figure 5 exclude irrelevant non-mutated cells. Surprisingly, ectopic expression of mDUT or vDUT in adoptive SWHEL B cells increased mutation of A:T base pairs relative to G:C base pairs (Table 1), a finding inconsistent with the dUTP-incorporation hypothesis. The inability of ectopic DUT expression to eliminate A:T mutation was independent of selection for HEL-binding, because abundant A:T mutation was evident in both exon and intron sequences from cells expressing ectopic DUT (Figure 6). Surprisingly, ectopic expression of mutant vDUT3X protein also increased A:T mutation relative to G:C mutation, in a manner very similar to expression of ‘wild-type’ vDUT (Table 1). Like wild-type vDUT, vDUT3X-expression also reduced the recovery of transduced cells from hosts (data not shown) and reduced the overall frequency of mutation similarly to wild-type vDUT (Table 1). This suggests that the affect of vDUT on cell survival or proliferation and mutation was largely independent of its dUTPase catalytic activity symbols), or mock-exposed (black symbols) to 37.5 ng purified vDUT (23) at 37°C for 2 h, prior to serial dilution and use in primer extension reactions. (C and D) Histograms indicate mean percentage ± SEM of 100 pmol of (C) dUTP, or (D) dTTP degraded in a 60 min pre-incubation with normalized limiting quantities of bead-bound fusion protein, prior to primer extension. (E) Mean percentage ± SEM of 100 pmol of dUTP degraded by bead-bound protein equivalent to 2.5 × 10⁵ G1- or S/G2-phase 3T3 cells or 3T3 cells transduced to express mDUT-GFP (details in Supplementary Figure S3).
and perhaps due to its immuno-modulatory properties (30). This does not necessarily imply that the effect of ectopic mDUT on mutation was also independent of its dUTPase activity, because mDUT and vDUT are structurally unrelated proteins (23). We did not experiment with an mDUT mutant because, unlike vDUT, mammalian dUTPase holoenzyme is trimeric (31). Since dUTPase is an essential protein in yeast (32), incorporation of mutant mDUT into endogenous trimers had potential to produce an uninterpretable phenotype.

Figure 5. Histograms showing the mean number of point mutations (±SEM) at G:C or A:T base pairs per mutated SWHEL IgH sequence recovered by single cell PCR from GFP+ cells, 6 or 7 days after adoptive transfer. The sequence window is illustrated by Figure 6. The number of sequences contributing to each mean is indicated by the number, n. Cells were transduced to express GFP plus the protein indicated. Control cells expressed GFP alone. Transitions (ts', dark grey) and transversions (tv', light grey) at G:C base pairs are shown as stacked histograms;—note that the mean no. of G:C transversions in cells transduced with ugi was zero (Table 1). All mutations (i.e. transitions and transversions) at A:T base pairs are indicated by the white histograms. White asterisks are placed on histograms where the mean was significantly different (P < 0.05) from the control mean by non-parametric ANOVA.

Figure 6. The distribution of mutations at G:C and A:T base pairs across SWHEL alleles from MSH2-proficient B cells. (A) Mirrored mutation profiles for (top) cells expressing ugi plus GFP, or GFP alone, versus (bottom) cells expressing vDUT-GFP fusion protein, vDUT plus GFP or mDUT plus GFP. Histograms represent the number of mutations detected at a particular (black) G:C base pair or (white) A:T base pair. The black/white ‘bar code’ at bottom shows the distribution of G:C and A:T base pairs in the sequenced region of the germline SWHEL IgH allele, counting from the start ATG codon as nucleotide 1–3. The scale diagram below the skylines indicates the positions of coding (shaded) and non-coding (thin line) regions, including CDRs [IMGT definition (41)]. Arrows indicate 5’ primers used for nested PCR amplification and the 3’ primer used for sequencing (the 3’-PCR primers are off-scale). The location of a 562-bp germline deletion characteristic of the SWHEL allele (20) is shown by ‘Δ’.

The minor UNG-mediated pathway of A:T mutation is also not ablated by ectopic dUTPase

Although ectopic DUT expression did not reduce A:T mutation in ‘wild-type’ SWHEL B cells, it remained possible that MSH2-independent A:T mutation might be sensitive to ectopic DUT expression. We therefore transduced msh2−/− SWHEL B cells to express GFP, vDUT and GFP, mDUT and GFP or vDUT3X and GFP. vDUT-expression in msh2−/− cells appeared to be particularly cytotoxic or anti-proliferative, because very few GFP+ cells were recoverable from hosts 7 days after adoptive transfer (data not shown). We therefore analysed mutation in transduced msh2−/− SWHEL B cells after only 6 days in adoptive hosts. Adoptive SWHEL cells appeared to rely on MSH2 protein for A:T mutation substantially more than reported for Peyer’s patch B cells [reviewed in (1)]. This meant that only seven mutations at A:T were detected in 80 mutated sequences from GFP-transduced msh2−/− SWHEL B cells (Table 1). Transduction of msh2−/− SWHEL B cells with mDUT, vDUT or vDUT3X had no significant effect on A:T mutation in msh2−/− cells.
For instance, amplification of J-CH intron DNA from conventional pooled DNA amplification is used (1,34). That is, every mutation can be assigned with certainty from PCR amplification of DNA from pooled cells. PCR, and thus avoids multiple artefacts which arise or bone marrow-transgenic mice, and transduced adoptive B cells acquire mutations in an otherwise wild-type milieu. Furthermore, our methodology replaces cloning of bulk PCR products with single-cell cloning, but no such cross-over is detected when single cell PCR is used, and the mutations present in each cell are never erroneously counted twice. Similar precision is impossible when conventional pooled DNA amplification is used (1,34). For instance, amplification of J-Ch intron DNA from bulk DNA of F1 mice results in cross-over between IgH SNPs in many of the sequences recovered by cloning, but no such cross-over is detected when single cell PCR is used [(33); K.K.E.Lau and C.J.Jolly, unpublished data]. Inheritance of common mutations by clonally-related daughter cells can affect data collected by single cell PCR, as it does data collected by any other method, but many founder cells contribute to the mutating pool in our system (we transfer ≥10⁴ HEL-reactive cells), co-occurrence of two or more mutations in cells from the same host [a working definition of ‘clonality’ (35)] represented a mere 6/856 mutations (0.7%) in our msh2+/+ mutation database (these mutations are highlighted in Supplementary Figure S4). MSH2-deficiency greatly increased the accumulation of mutations at hotspots, causing more cells to share mutations, as noted before (35). However, the same hotspots occurred repeatedly in independent experiments, proving that the increased hotspot focus arose from preferential targeting and not from clonal expansion (Supplementary Figure S4). We did not prune our databases of ‘clonal’ mutations, which inevitably over-compensates for clonality (35), because our msh2+/+ database demonstrated that clonality was a minor issue in our experimental system. Selection must affect the mutations that are tolerated in the SWHEL IgH exons, but antigen selection would have been uniform in all of our experiments. Furthermore, we showed that ectopic dUTPase did not suppress A:T mutation in exon or intron sequences (Figure 6).

DISCUSSION

Advantages of the transduction/adoptive transfer model and single-cell PCR

The established technique to detect perturbation of Ig SHM in DNA repair-defective mice is sequencing of endogenous IgH genes amplified from activated Peyer’s patch B cells of middle-aged transgenic or gene-targeted mice (33). Our new transduction/adoptive transfer method bypasses the need to make germ-line- or bone marrow-transgenic mice, and transduced adoptive B cells acquire mutations in an otherwise wild-type milieu. Furthermore, our methodology replaces cloning of bulk PCR products with single-cell PCR, and thus avoids multiple artefacts which arise from PCR amplification of DNA from pooled cells. That is, every mutation can be assigned with certainty to an individual cell when single cell PCR is used, and the mutations present in each cell are never erroneously counted twice. Similar precision is impossible when conventional pooled DNA amplification is used (1,34). For instance, amplification of J-Ch intron DNA from bulk DNA of F1 mice results in cross-over between IgH SNPs in many of the sequences recovered by cloning, but no such cross-over is detected when single cell PCR is used [(33); K.K.E.Lau and C.J.Jolly, unpublished data]. Inheritance of common mutations by clonally-related daughter cells can affect data collected by single cell PCR, as it does data collected by any other method, but many founder cells contribute to the mutating pool in our system (we transfer ≥10⁴ HEL-reactive cells), co-occurrence of two or more mutations in cells from the same host [a working definition of ‘clonality’ (35)] represented a mere 6/856 mutations (0.7%) in our msh2+/+ mutation database (these mutations are highlighted in Supplementary Figure S4). MSH2-deficiency greatly increased the accumulation of mutations at hotspots, causing more cells to share mutations, as noted before (35). However, the same hotspots occurred repeatedly in independent experiments, proving that the increased hotspot focus arose from preferential targeting and not from clonal expansion (Supplementary Figure S4). We did not prune our databases of ‘clonal’ mutations, which inevitably over-compensates for clonality (35), because our msh2+/+ database demonstrated that clonality was a minor issue in our experimental system. Selection must affect the mutations that are tolerated in the SWHEL IgH exons, but antigen selection would have been uniform in all of our experiments. Furthermore, we showed that ectopic dUTPase did not suppress A:T mutation in exon or intron sequences (Figure 6).

Negation of the ‘dUTP-incorporation’ model of AID-induced A:T mutation

We used our transduction/adoptive transfer model to test the intriguing hypothesis that incorporation of dUTP opposite adenine bases during G1-phase long patch excision repair is the mechanism by which AID induces mutation of A:T base pairs in Ig V genes (1). Ectopic expression of active mouse and viral dUTPase in the nucleus of G1-phase cells did not significantly reduce mutation at A:T base pairs in Ig V genes in vivo. Although we cannot be certain that G1-phase expression of nuclear dUTPase reduces G1-phase nuclear dUTP levels, it is probable that it does for the following reasons. vDUT-GFP extracted from G1-phase cells was active (Figure 4E), and it is unlikely that a non-covalent inhibitor of dUTPase could suppress both mDUT and vDUT function, because the structure and surface epitopes of these two enzymes are quite dissimilar (23). Finally, in natural infections EBV expresses vDUT mostly highly in non-dividing (i.e. G1-phase) cells (37), where vDUT presumably functions to degrade dUTP in the nucleus. We therefore conclude that dUTP-incorporation is unlikely to be involved in the mechanism of AID-induced mutation of A:T base pairs induced by AID via either mismatch repair or UNG, lending credence to the notion that AID-induced A:T mutation is directly due to the low fidelity of pol η (6).

An alternative model for recruitment of pol η to mutating antibody genes

Although both vDUT and mDUT caused significant changes in Ig mutation frequencies, they did not cause the same changes, and the changes induced did not necessarily depend on dUTPase activity, so the mechanism is unknown. It is possible that the changes we observed are due to statistical error. Nonetheless, it is worthwhile to consider what the observed changes might indicate. Ectopic mDUT increased mutation at A:T base pairs in msh2+/+ cells, but not in msh2−/− cells (Figure 5), suggesting that it induced an MSH2-dependent A:T mutation process, as does AID. Since endogenous mDUT is abundant in S-phase cells (17–19), it seems likely that ectopic mDUT exerts its effects on antibody mutation outside S-phase. If this is so, our data are consistent with Neuberger et al.’s (1) proposal that MSH2 processes U:G base pairs in antibody genes outside S-phase, even though our data do not support a role for dUTP-incorporation in producing mutations. Why, though, should a translesion polymerase such as pol η be recruited to synthesize DNA in an excision patch induced by a U:G mismatch? Both U or G provide instructive templates for DNA pol δ, the polymerase recruited by classical post-replication mismatch repair (12). We propose a novel possibility—stalling of DNA synthesis as a consequence of inappropriate nucleotide pools during G1-phase mismatch repair. Direct measurement of dNTP concentrations in (inherently leaky) nuclei of germinal centre B cells is not feasible, because it requires cold fractionation of organelles from very large numbers of cells using non-aqueous methods. However, the nuclear concentrations of dNTPs are 1.8× (dTTP) to 9× (dGTP)
lower in the nucleus of G1-phase compared to S-phase CHO cells (38). Low concentrations of substrate dNTPs during G1-phase AID-induced mismatch repair could reduce the fidelity of DNA pol η, inducing further mismatch repair, and could also explain the recruitment of DNA pol η in place of pol δ, because pol δ could stall in the presence of sufficiently low dNTP concentrations. Stalling could potentiate mono-ubiquitination of PCNA and recruitment of error-prone pol η—an enzyme apparently adapted to function outside S-phase—in place of pol δ (16,39,40).

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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