Altered spectra of hypermutation in DNA repair-deficient mice

David B. Winter and Patricia J. Gearhart*

Laboratory of Molecular Genetics, National Institute on Aging, National Institutes of Health, Baltimore, MD 21224-6825, USA

Affinity maturation of the humoral immune response is based on the ability of immunoglobulin variable genes to undergo a process of rapid and extensive somatic mutation followed by antigenic selection for antibodies with higher affinity. While the behaviour of this somatic hypermutation phenomenon has been well characterized over the last 20 years, the molecular mechanism responsible for inserting mutations has remained shrouded. To better understand this mechanism, we studied the interplay between hypermutation and other DNA associated activities such as DNA repair. There was no effect on the frequency and pattern of hypermutation in mice deficient for nucleotide excision repair, base excision repair and ataxia-telangiectasia mutated gene repair of double strand breaks. However, variable genes from mice lacking some components of mismatch repair had an increased frequency of tandem mutations and had more mutations of G and C nucleotides. These results suggest that the DNA polymerase(s) involved in the hypermutation pathway produces a unique spectra of mutations, which is then altered by mismatch repair and antigenic selection. We also describe the differential pattern of expression of some nuclear DNA polymerases in hypermutating versus non-hypermutating B lymphocytes. The rapidly dividing germinal centre B cells expressed DNA polymerases alpha, beta, delta, epsilon and zeta, whereas the resting non-germinal centre cells did not express polymerases alpha or epsilon at detectable levels, although they did express polymerases beta, delta and zeta. The lack of expression of polymerase epsilon in the non-germinal centre cells suggests that this enzyme has a critical role in chromosomal replication but does not participate in DNA repair in these cells.

Keywords: nucleotide excision repair; base excision repair; mismatch repair; ataxia-telangiectasia mutated; DNA polymerases

1. INTRODUCTION

The complex genome of vertebrates requires stability and regulation to ensure that mutation and damage do not destroy the integrity of the genetic code. While some forms of genomic instability, such as microsatellite instability or transposons, appear unregulated and may damage the genome, instability of the immunoglobulin gene loci is tightly controlled and is essential to diversify the immune response. These genes undergo (i) rearrangement of gene segments to produce a plethora of variable (V) regions required for the humoral immune response; (ii) gene conversion (in some species) to increase the diversity of V regions; (iii) heavy chain class switching of constant genes to produce antibodies with different effector functions; and (iv) somatic hypermutation of V genes to generate tremendous increases in affinity for antigens.

Somatic hypermutation is an incredible phenomenon that draws the curiosity of a wide variety of researchers from immunologists, geneticists and biochemists to medical clinicians and evolutionary biologists. This intense interest is due to the ability of hypermutation to compress a million years' worth of normal evolutionary

selection into the space of a few weeks by a process of high-speed mutation and antigenic selection. These mutations are inserted into rearranged V genes by an unknown molecular mechanism after antigen stimulation. B lymphocytes expressing the mutated genes then compete for binding to antigen. Those cells with immunoglobulin receptors with high affinity are restimulated by antigen and survive, and cells with low affinity are not stimulated and die by apoptosis. This results in an overall increase in the ability of the humoral immune system to bind, neutralize and destroy invading organisms.

Since hypermutation was first described 20 years ago, the phenomenon has been well characterized. It has been demonstrated that hypermutation (i) only occurs after T-cell-dependent antigen stimulation; (ii) takes place once B cells reach germinal centres in secondary lymphoid tissues; (iii) inserts mutations randomly, with the exception of a few hot spots, within a 2 kb region around the rearranged V gene; and (iv) targets all four bases for mutation at similar frequencies with transitions more common than transversions (Kim *et al.* 1981; Pech *et al.* 1981; Selsing & Storb 1981; Gearhart & Bogenhagen 1983; Golding *et al.* 1987; Lebecque & Gearhart 1990; Smith *et al.* 1996; Wilson *et al.* 1998). However, while the nature of the phenomenon has been well characterized, the causative molecular mechanism—the protein machinery—remains completely shrouded.

*Author for correspondence (gearhartp@grc.nia.nih.gov).

Figure 1. DNA repair pathways. (a) Nucleotide excision repair. Thymine dimers are recognized by XPC, XPA and RPA proteins, and form a multi-protein complex with TFIIH and the excision nucleases ERCC1/XPF and XPG. The resultant gap is filled in by a DNA polymerase. (b) Base excision repair. 8-oxoguanine is excised by OGG1 glycosylase, and the apurinic site is removed and filled in by a DNA polymerase. (c) Mismatch repair. MSH2 and MSH6 proteins recognize single base mismatches and form a complex with PMS2 and MLH1. The mispair is excised and the gap is repaired by a DNA polymerase.

General DNA molecular mechanisms, such as replication, repair and transcription, as well as immunoglobulin-specific mechanisms, such as recombination and isotype switching, require and sometimes share similar machinery, such as helicases, nucleases, polymerases and ligases. It is speculated that hypermutation, therefore, also requires such proteins and may share some with other pathways. Since hypermutation inserts incorrect sequences into the genome, we examined the interactions between hypermutation and DNA repair under the hypothesis that DNA repair is involved in hypermutation in one of three ways.

- (i) An error-prone repair mechanism generates the mutations (Brenner & Milstein 1966).
- (ii) Repair is suppressed in V genes, which allows natural errors to accumulate.
- (iii) Repair recognizes the mutations and competes with hypermutation to repair them.

We have addressed this question by examining the ability of DNA repair-deficient mice to mutate their V genes after antigenic stimulation.

2. DNA REPAIR PATHWAYS

There are three major DNA excision repair pathways—nucleotide excision repair (NER), base excision repair (BER) and mismatch repair (MMR). While each of these pathways is responsible for the repair of specific types of damage, they share a similar pattern of recognizing and repairing DNA damage. This includes recognition of a target site, recruitment of repair proteins in a stepwise manner, excision of a region around the site, filling in the resulting gap by use of specific polymerases and finally ligating the DNA ends together. The molecular mechanism of each repair pathway consists of a unique multi-protein machine, although some protein components, such as

DNA polymerases, are shared by the different pathways (figure 1).

By studying the immune response in mice deficient in critical steps of each of these pathways, it may be possible to identify components of the hypermutation machinery and determine how hypermutation and DNA repair mechanisms interact. Our experimental approach was to immunize wild-type and various DNA repair-deficient mice with a well-characterized antigen, phenyl-oxazolone coupled to chicken serum albumin (phOx-CSA), separate germinal centre B cells by flow cytometry, and clone and sequence the V_κOx1 light chain gene, which is used in the anti-phOx response (Kaartinen *et al.* 1983). The frequencies and patterns of mutations were then compared between the wild-type and mutant mice. Mutations at codons 34 and 36 were ignored for all groups as these are highly selected mutations in V_κOx1 antibodies after immunization with phOx-CSA and might bias the data (Griffiths *et al.* 1984; Alzari *et al.* 1990).

(a) *Hypermutation in nucleotide excision repair-deficient mice*

The NER pathway is responsible for repairing DNA that is damaged by ultraviolet irradiation, bulky lesions and strand cross-linking. Deficiencies in proteins of the NER pathway are responsible for the human diseases of xeroderma pigmentosum (XP) and Cockayne's syndrome. Mice deficient in the *XPA* gene (figure 1a) are susceptible to ultraviolet- and chemically induced skin tumours and are unable to repair dipyrimidine photoproducts (Nakane *et al.* 1995).

Comparison of V_κOx1 sequences between phOx-CSA immunized wild-type and *XPA*-deficient mice revealed that there was no change in frequency or distribution of mutations in mice lacking the NER pathway (figure 2) (Winter *et al.* 1998). This rules out a role for NER in generating mutations during error-prone repair. There

Figure 2. Frequency and distribution of mutations in the rearranged $V_{\kappa}Ox1$ gene from DNA repair-deficient mice. A diagram of the flanking and coding regions that were sequenced is depicted at the bottom of the figure with the complementarity-determining regions in black boxes. Numbers on the abscissa refer to the nucleotide distance, with '1' representing the first base in the V gene and negative numbers showing the 5' flanking region. (a) C57BL/6, 1.4% mutations per base pair; (b) $Xpa^{-/-}$, 1.5%; (c) $Msh2^{-/-}$, 1.3%; (d) $Pms2^{-/-}$, 0.9%; (e) $Mlh1^{-/-}$, 0.7%.

was also no change in the pattern of mutations (table 1), which one might expect if NER was involved in competing with hypermutation to repair the mutational errors. This finding is not surprising since NER is not

normally involved in repairing mismatches. These conclusions are supported by the findings of others who have looked at various XP protein-deficient mice or humans and found no significant decrease in their ability to hypermutate V genes (Wagner *et al.* 1996; Kim *et al.* 1997; Shen *et al.* 1997; Jacobs *et al.* 1998).

(b) *Hypermutation in base excision repair-deficient mice*

The BER pathway corrects small non-bulky lesions, oxidative damage and abasic sites. There was a report demonstrating an increase in a BER glycosylase, OGG1, in human germinal centre B cells (Kuo & Sklar 1997). It is possible that the increased cell division rate of germinal centre B cells results in increased oxidative stress and produces 8-oxoguanine. The OGG1 glycosylase enzyme removes 8-oxoguanine to produce an abasic site, which is then replaced by an undamaged residue (figure 1b). In the absence of OGG1, 8-oxoguanine remains and preferentially pairs with mismatched dATP to generate a G:C to T:A mutation. Using mice deficient in OGG1 (Klungland *et al.* 1999), we determined that they retain the ability to hypermutate their V genes and show no change in pattern of mutation (Winter & Gearhart 2001). These results and those of others rule against a role of BER in hypermutation (Jacobs *et al.* 1998).

(c) *Hypermutation in mismatch repair-deficient mice*

MMR is used during DNA replication to correct mismatches due to polymerase error. Because hypermutation apparently results in mismatched bases at some point in the process, it is intuitive that these two mechanisms must somehow interact, either in cooperation or in competition with each other. If the two systems cooperate, that is mutations are introduced during the process of MMR, then mice deficient in MMR should have a dramatic decrease in the frequency of hypermutation. If the two systems compete, that is MMR attempts to remove mismatches introduced by hypermutation, then MMR-deficient mice should demonstrate a change in the pattern of mutations due to a lack of correction of mismatches.

We examined the frequency and pattern of mutations in mice deficient for three MMR proteins: MSH2, PMS2 and MLH1 (figure 1c). Mice deficient in MSH2, which recognizes mismatches, cannot repair single base mismatches, have unstable microsatellite sequences and have an increase in lymphoid tumours (Umar *et al.* 1994; Reitmaier *et al.* 1995, 1997; De Wind *et al.* 1997; Cranston *et al.* 1997; Andrew *et al.* 1998). Comparison of $V_{\kappa}Ox1$ sequences between $Msh2^{-/-}$ and wild-type mice immunized with pHx-CSA showed a similar frequency and distribution of mutations in V genes, showing that MMR does not generate hypermutations (figure 2) (Phung *et al.* 1998). However, the pattern of mutations changed dramatically. In wild-type mice, mutations are distributed fairly evenly among the four bases, but in the $Msh2^{-/-}$ mice, over 90% of the mutations occurred at G:C bp (table 1a). These results suggest that the hypermutation pathway preferentially inserts mutations at G:C bp and/or an MSH2-dependent pathway preferentially repairs mismatches at G and C. This is the first evidence

Table 1. *Altered spectra of mutations in Msh2^{-/-} and Pms2^{-/-} clones*(a) Types of substitutions in V_κOx1 genes (mut, mutations)

substitution		C57BL/6 92 mut %	<i>Xpa</i> ^{-/-} 123 mut %	<i>Msh2</i> ^{-/-} 108 mut %	<i>Pms2</i> ^{-/-} 76 mut %	<i>Mlh1</i> ^{-/-} 74 mut %	<i>Atm</i> ^{-/-} 45 mut %
A to	G	19	19	0	17	8	13
	T	16	8	5	8	12	11
	C	11	6	1	8	3	2
T to	C	9	8	1	7	7	13
	A	2	7	1	4	5	2
	G	1	2	1	5	5	11
C to	T	10	17	25	12	19	16
	A	2	3	5	3	8	2
	G	2	2	1	9	3	2
G to	A	17	19	44	19	20	20
	T	4	3	5	1	2	4
	C	7	6	11	7	8	2

(b) Tandem mutations comparing observed and expected values

mice	mutations (clones)	tandems		<i>p</i> -value
		observed	expected	
C57BL/6	106 (12)	4	2.46	0.48
<i>Xpa</i> ^{-/-}	137 (16)	11	3.69	0.02
<i>Msh2</i> ^{-/-}	135 (22)	3	1.84	0.56
<i>Pms2</i> ^{-/-}	128 (30)	11	1.19	< 10 ⁻⁶
<i>Mlh1</i> ^{-/-}	81 (17)	3	1.22	0.25

that MMR is active around immunoglobulin V genes during hypermutation and that the repair mechanism is in direct competition with the mutation mechanism.

If MMR cooperates with hypermutation, then removal of the excision step of the repair pathway should also affect the frequency of mutations. In MMR, this step relies on two proteins, PMS2 and MLH1 (figure 1c). Mice deficient in either of these proteins exhibit microsatellite instability, an increase in tumours and a complete lack of mismatch repair (Baker *et al.* 1995, 1996; Edelmann *et al.* 1996). As with the *Msh2*^{-/-} mice, *Pms2*^{-/-} and *Mlh1*^{-/-} mice are able to hypermutate their V genes, although at only about 50% of the level of wild-type mice (figure 2) (Winter *et al.* 1998; Phung *et al.* 1999). This may be due to a decreased viability of the cells because of their rapid rate of division without the ability to repair replication errors efficiently. The pattern of mutation also changed in *Pms2*^{-/-} but not in *Mlh1*^{-/-} mice. *Pms2*^{-/-} mice showed a significant increase in tandem mutations (two mutations in a row) over wild-type or other repair-deficient mice (*p* < 10⁻⁶) (table 1b). Adjacent or clustered mutations may be generated by an error-prone DNA polymerase during short patch repair (Gearhart & Bogenhagen 1983) and preferentially removed during PMS2-dependent repair. This supports our earlier conclusion that MMR is active around immunoglobulin genes during hypermutation and competes with the mutation mechanism to repair mismatches. Some of the newly described bypass polymerases are good candidates for the hypermutation polymerase since they frequently misincorporate

consecutive nucleotides on undamaged DNA (Woodgate 1999). Other laboratories that have examined immunized mice that are deficient in proteins of the MMR pathway have supported most of our conclusions (Frey *et al.* 1998; Jacobs *et al.* 1998; Kim *et al.* 1999; Rada *et al.* 1998).

(d) *Hypermutation in ataxia-telangiectasia mutated-deficient mice*

There is great interest and research on the immunoglobulin loci as the targets of two other types of regulated genomic instability: (i) recombination of V_H diversity, and joining gene segments, and (ii) heavy chain class switching. Both of the latter events break apart the chromosome with single and double strand breaks and rejoin sections together. We therefore speculated that hypermutation at immunoglobulin loci may involve single and/or double strand breaks if bases are removed and replaced by an error-prone polymerase. To test what interactions may occur between double strand break repair and hypermutation, we examined the ability of double strand break repair-deficient mice to mutate their V genes after antigen stimulation.

The ataxia-telangiectasia mutated (ATM) gene codes for a PI3-kinase involved in multiple pathways, including DNA double strand break repair and cell cycle checkpoint control (reviewed in Brown *et al.* 1999). Humans bearing defects in this gene exhibit cellular and humoral immune deficiencies, susceptibility to ionizing radiation damage and increased lymphomas. An *Atm*^{-/-} mouse model exhibits similar deficiencies as well as defects in cell cycle control.

Immunized *Atm*^{-/-} mice (obtained from R. Hodes, National Institutes of Health, Bethesda, MD, USA) had a frequency of 0.6% mutations per base pair in their V_κOx1 genes (table 2), which is similar to the levels found in wild-type mice. The types of substitutions were also similar between *Atm*^{-/-} and wild-type mice (table 1a), suggesting that the ATM-dependent double strand break repair pathway is not involved in hypermutation and the cell cycle machinery does not strictly control the mutation mechanism.

Table 2. *Mutations in V_κOx1 genes from ATM-deficient mice*

(Total of 18 mutated clones (8388 bp sequenced); 54 mutations; 0.6% mutations per base pair.)

clone	number	position ^a
22	1	A27T
28	1	G77A
34	1	G224A
50	1	A87T
66	1	T-169Δ
36	2	C97A, A104T
55	2	C97A, A156G
74	2	G89A, T273C
132	2	C78T, A156G
73	3	G194T, C210T, C212T
109 ^b	3	G37A, A107C, G175A
156	3	T-97A, C116T, A190G
162	3	A39T, G102A, G247A
166	3	A-56T, G25C, A107G
25 ^b	4	C66T, G101A, C210A, C212T
5	5	T-23C, T-11G, T90G, C97A, A104T
147	8	T-108C, T-88G, T-85G, T-35C, T90C, C97A, G175A, C210T
18	9	T-107C, G-87T, A-72T, T-53G, A-34G, C97A, A104T, C123G, A146G

^a Data show the original nucleotide, position of the nucleotide and the mutated nucleotide. Nucleotide position is numbered according to Winter *et al.* (1998); negative numbers represent mutations in the 5' intron. Δ deletion.

^b Join is out of frame.

Table 3. *Eukaryotic DNA polymerase subunits examined*

DNA polymerase	function	subunits
Pol α	DNA replication	catalytic p180, associated p68
Pol β	base excision repair	catalytic p39
Pol δ	DNA replication and repair	catalytic p125, associated p48
Pol ε	DNA replication and repair	catalytic p250, associated p55
Pol ζ	translesion synthesis	catalytic p353

3. DNA POLYMERASES

Whatever the mechanism, it is intuitive that a DNA polymerase inserts mismatched bases during hypermutation. There are several well-defined nuclear DNA polymerases that are involved in replication and repair (table 3) (Wang 1996; Wood & Shivji 1997). To determine if any of these are expressed in B cells undergoing hypermutation, mice were immunized with phOx-CSA, and germinal centre (hypermutating) and non-germinal centre (non-hypermutating) B cells were separated by flow cytometry (Kelsoe 1996). The expression of subunits of each of the major nuclear DNA polymerases was determined by reverse transcriptase-PCR (table 4) (Winter *et al.* 2000). Comparison of RNA expression of the polymerases between the germinal centre and non-germinal centre B cells showed that polymerases alpha, beta, delta, epsilon and zeta were expressed in the mutating cell population. On the other hand, polymerase alpha and epsilon subunits were not expressed in the resting non-germinal centre B cells, although they did express polymerases beta, delta and zeta. Thus polymerases alpha

and epsilon appear to have a major role in chromosomal replication. The lack of expression of polymerase epsilon in the resting cells indicates that this enzyme is not used in any DNA repair pathways by these cells. The expression of polymerase zeta by resting cells suggests that it has another role in DNA repair, perhaps in recombination, in addition to its function of bypassing damage during chromosomal replication.

4. CONCLUSIONS

Efficient DNA repair is essential for cell survival in the face of continuous assault by DNA damaging agents from the environment and cellular processes. From bacteria to mammals, DNA repair pathways have been preserved, diversified and backed up with redundant pathways. These pathways are so efficient that, working together, they ensure that mutations are rarely fixed into the genome during replication ($< 10^{-8}$ mutations per base pair).

In the face of such efficient repair, how can a mechanism, such as somatic hypermutation, evolve to target a set of genes for a millionfold increase in mutations? Hypermutation is present in most vertebrates and appears to be vigorously active even in ancient orders such as *Chondrichthyes*. It could have evolved during suppression of repair in V genes, or, conversely, during the process of error-prone repair. On the other hand, hypermutation may have evolved in competition with DNA repair, which suggests that repair helps to shape the final form of the mutations depending upon which mutations it most readily repairs.

Our results, along with the results of others, demonstrate that each of the three major DNA excision repair mechanisms may be disabled without disrupting hypermutation. It therefore seems unlikely that the mutations are the direct manifestation of error-prone repair of damaged DNA (Frey *et al.* 1998; Jacobs *et al.* 1998; Phung

Table 4. *Expression of DNA polymerases in PNA⁻ and PNA⁺ B cells*

(—, no expression after 60 rounds of nested PCR; +, strong expression after PCR amplification.)

experiment no.	PNA ⁻				PNA ⁺			
	1	2	3	4	1	2	3	4
Pol α catalytic	—	—	—	—	+	+	+	+
Pol α associated	+	+	+	+	+	+	+	+
Pol β catalytic	+	+	+	+	+	+	+	+
Pol δ catalytic	+	+	+	+	+	+	+	+
Pol δ associated	+	+	+	+	+	+	+	+
Pol ϵ catalytic	—	—	—	—	+	+	+	+
Pol ϵ associated	—	—	—	—	+	+	+	+
Pol ζ catalytic	+	+	+	+	+	+	+	+

et al. 1998, 1999; Rada *et al.* 1998; Winter *et al.* 1998, Kim *et al.* 1999; Wiesendanger *et al.* 2000). However, it is clear that inactivation of the MMR pathway produces changes in the spectra of mutations. This would only occur if MMR is active around V genes during hypermutation and is trying to correct the mismatches that arise during the mutational process. While MMR is ultimately overwhelmed by the sheer number of mutations, it appears to have an impact on the final phenotype of mutations, which includes a decrease in the frequency of tandem mutations and a change in the distribution of mutations among the four bases.

In addition to defining the interactions between DNA excision repair and hypermutation, we have shown here that there is little or no interaction between the ATM-dependent pathways of double strand break repair or cell cycle checkpoint control with hypermutation. Neither the frequency of mutations nor the pattern of mutations change significantly in *Atm*^{-/-} mice compared with wild-type mice. This does not preclude the possibility that other proteins within this or other DNA repair pathways are shared with the hypermutation pathway. It is likely that some of the ligases, helicases or polymerases used in repair are also involved in hypermutation. Indeed, the goal of testing for the expression of polymerases in germinal centre B cells was to determine which polymerases were potential candidates for use in hypermutation. As we have established, all five well-characterized nuclear DNA polymerases, including polymerase zeta, are expressed in hypermutating B cells (Winter *et al.* 2000). We will continue to study error-prone polymerases as a first step to understanding the hypermutation pathway because once a single protein is identified, the rest of the pathway is likely to fall quickly into place.

It is a pleasure to acknowledge the continued support of Vilhelm Bohr. We thank Richard Hodes for supplying *Atm*^{-/-} mice.

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