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Adams, C.L. and Macleod, M.K.L. and Milner-White, E.J. and Aitken, R. and Garside, P. and Stott, D.I. (2003) Complete analysis of the B-cell response to a protein antigen, from *in vivo* germinal centre formation to 3-D modelling of affinity maturation. *Immunology* 108(3):pp. 274-287.

<http://eprints.gla.ac.uk/3653/>

1 **Complete analysis of the B-cell response to a protein antigen, from *in vivo* germinal centre**
2 **formation to 3-D modelling of affinity maturation**

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1 **Summary**

2 Somatic hypermutation of immunoglobulin variable region genes occurs within germinal centres
3 (GCs) and is the process responsible for affinity maturation of antibodies during an immune
4 response. Previous studies have almost exclusively focused on the immune response to haptens,
5 which may be unrepresentative of epitopes on protein antigens. In this study, we have exploited a
6 model system that uses transgenic B- and CD4+ T-cells specific for hen egg lysozyme (HEL) and a
7 chicken ovalbumin peptide, respectively, to investigate a tightly synchronised immune response to
8 protein antigens of widely differing affinities, thus allowing us to track many facets of the
9 development of an antibody response at the antigen-specific B-cell level in an integrated system *in*
10 *vivo*.

11 Somatic hypermutation of immunoglobulin variable genes was analysed in clones of
12 transgenic B-cells proliferating in individual GCs in response to HEL or the cross-reactive low
13 affinity antigen, duck egg lysozyme (DEL). Molecular modelling of the antibody-antigen interface
14 demonstrates that recurring mutations in the antigen-binding site, selected in GCs, enhance
15 interactions of the antibody with DEL. The effects of these mutations on affinity maturation are
16 demonstrated by a shift of transgenic serum antibodies towards higher affinity for DEL in DEL-
17 cOVA immunized mice. The results show that B-cells with high affinity antigen receptors can
18 revise their specificity by somatic hypermutation and antigen selection in response to a low
19 affinity, cross-reactive antigen. These observations shed further light on the nature of the immune
20 response to pathogens and autoimmunity and demonstrate the utility of this novel model for
21 studies of the mechanisms of somatic hypermutation.

22

23

1 **Introduction**

2 Following antigen challenge, antigen-specific B-cells enhance the “quality”, i.e. specificity
3 and affinity, of the antibodies they produce over time. This process is important for protective
4 immunity to pathogens and defects in this process lead to a profound susceptibility to bacterial
5 infections ^{1,2}.

6 This improvement in antibody affinity and specificity takes place in the microenvironment
7 of the germinal centre (GC) by the process of affinity maturation of B-cell antigen receptors ³⁻⁵.
8 Firstly, rearranged immunoglobulin (Ig) variable (V) region genes are randomly mutated in
9 centroblasts within the dark zone of the GC by a process known as somatic hypermutation ⁶⁻⁹.
10 Surface expression of these mutated Igs is upregulated during differentiation of the centroblasts to
11 centrocytes, which migrate to the light zone. Here they are pre-programmed to die by apoptosis
12 unless they receive survival signals by ligation of the B-cell receptor (BCR) with antigen which they
13 encounter on the surface of follicular dendritic cells (FDCs). Competition for antigen results in
14 selection of B-cells with high affinity receptors. The surviving B-cells receive further signals from
15 CD4⁺ helper T-cells via their CD40 ligand and differentiate into either long-lived memory cells or
16 antibody-producing plasma cells ^{3,10}.

17 Basic features of the hypermutation process have been deduced from the sequences of
18 antibodies recovered from different stages of immune responses to a variety of antigens and haptens
19 ¹¹. Single nucleotide substitutions are introduced in a stepwise manner into the Ig V region genes at
20 an estimated rate of one mutation per thousand base pairs per generation ⁶. Mutations occur in both
21 DNA strands ¹² with a strong bias for transitions over transversions ⁸ and the mutation machinery
22 targets particular sequence motifs, or “hot spots” of which the RGYW motif is best known ¹³⁻¹⁵.

1 Somatic hypermutation in GCs has primarily been studied in mice responding to haptens, in
2 which the response is dominated by single rearranged heavy and light chain V- genes¹⁶⁻¹⁸. It has
3 been difficult to examine the germinal centre response to protein antigens directly *in vivo* due to the
4 low precursor frequency and heterogeneity of B cells specific for a particular protein and the
5 presence of multiple epitopes on the antigen, resulting in a response comprising a very
6 heterogeneous population of rearranged V- genes¹⁹. In this study, we have exploited an adoptive
7 transfer mouse model²⁰ which uses antigen receptor transgenic (Tg) B- and CD4⁺ T-cells specific
8 for hen egg lysozyme (HEL) and a chicken ovalbumin (cOVA) peptide, respectively, to investigate
9 a tightly synchronised immune response to a protein antigen. Increasing the precursor frequency of
10 T and B lymphocytes to defined epitopes in this model has greatly facilitated studies on B-cell
11 differentiation and migration into the lymphoid follicle^{20, 21}, receptor-editing of anergic B-cells²²
12 and self/non-self B-cell discrimination^{23, 24}.

13 The MD4 mouse strain²³ carries rearranged heavy and light chain transgenes which encode
14 the HEL-specific monoclonal antibody, HyHEL-10, offering an unique opportunity to study B-cell
15 somatic hypermutation in germinal centres during the immune response to a protein antigen *in vivo*.
16 We have exploited the fact that the HyHEL-10 antibody has approximately three thousand times
17 lower affinity for duck egg lysozyme [$K_a = 1.3 \times 10^7 M^{-1}$ (²⁵)] compared to HEL ($K_a = 4.5 \times 10^{10} M^{-1}$)
18 but the MD4 Tg B-cells are still able to expand in response to this lower affinity antigen²⁶.

19 The availability of the high resolution 3-D structures of HyHEL-10, its antigen and the
20 antibody/antigen complex²⁷⁻²⁹ has allowed us to use molecular modelling to study the effects of
21 mutations in B-cell clones proliferating within developing GCs. Molecular modelling of the
22 HyHEL-10/DEL interface indicates that antigenic selection for mutations that enhance interactions
23 with DEL specific residues is taking place within the GCs of DEL-cOVA immunized recipients.

1 Selection of these mutations was accompanied by increased affinity of the transgenic antibodies for
2 DEL.

3 To our knowledge, this is the first time that selection of replacement mutations in GC B-
4 cells responding to a protein antigen has been observed directly *in vivo* and their effects on the
5 affinity of the antibody-antigen interaction followed through high resolution modelling of the
6 receptor/antigen complex. This study expands our understanding of the individual and cumulative
7 effects of somatic hypermutation on the binding of antibody to its epitope, leading to further
8 insight into how the immune system combats infectious diseases by refinement of the antibody-
9 antigen interaction during the evolution of the immune response.

10

11 **Methods and Materials**

12

13 *Preparation of DEL-cOVA and HEL-cOVA conjugates*

14 Lysozyme was prepared from domestic duck eggs following the method previously described by
15 Smith-Gill *et al*³⁰. It was free of contaminants as determined by SDS-PAGE and was shown to
16 contain high specific activity by the *Micrococcus lysodeikticus* cell turbidimetric assay³¹.

17 Duck egg lysozyme or hen egg lysozyme (Biozyme laboratories) was coupled to chicken
18 ovalbumin (Sigma) using glutaraldehyde [Sigma²⁰].

19

20 *Mouse strains, adoptive transfer and immunization*

21 D011.10 (BALB/c x C57BL/6) F₁ and MD4 (BALB/c x C57BL/6) F₁ transgenic (Tg) mice were
22 used. All mice were bred and maintained in a specific pathogen-free facility at the University of
23 Glasgow following home office regulations. D011.10 T-cells express a Tg TCR specific for chicken
24 ovalbumin peptide (323-329) in the context of 1-A^d³². Cell suspensions containing 1 - 2.5 x 10⁶

1 D011.10 CD4⁺ T-cells and 2.5 - 5 x 10⁶ MD4 B-cells were transferred intravenously into (BALB/c-
2 Igh^b x C57BL/6)F₁ recipients ²⁰. After one day, HEL-cOVA or DEL-cOVA conjugate (130µg) in
3 complete Freund's adjuvant (CFA) was injected subcutaneously on the back. The Tg TCR was
4 detected by the clonotypic monoclonal antibody KJ1-26 ³³. MD4 B-cells express the anti-HEL
5 HyHEL-10 monoclonal antibody with both IgM and IgD isotypes ²³ and can be identified using an
6 antibody specific for the IgM^a allotype. The percentage of CD4⁺ KJ1-26⁺ D011.10 T-cells and
7 B220⁺ IgM^{a+} MD4 B-cells from the transgenic donors was determined by flow cytometry.

8

9 *Immunohistochemistry/FACS*

10 The lymph nodes (brachial, axillary, cervical, inguinal, and para-aortic) were harvested at specific
11 time points; half of the lymph nodes from each recipient were used to prepare single cell
12 suspensions and stained for flow cytometry as previously indicated ²⁰. FACS data were collected
13 and analysed using FACScalibur (Becton Dickson) and the Cellquest software program,
14 respectively.

15 The remaining lymph nodes were frozen in liquid nitrogen in O.C.T. embedding medium
16 (Miles) and stored at -80°C. Tissue sections (6µM) were mounted on pre-coated slides (Speci-
17 microsystems, Surrey), fixed in acetone and stained with biotinylated anti-IgM^a, HEL, DEL, peanut
18 agglutinin (PNA) and anti-Thy1.2 as previously described ²⁰.

19

20 *Competitive ELISA*

21 All reagents were diluted in ED buffer (0.2% fetal calf serum, 0.05% Tween 20 in PBS) unless
22 stated otherwise. Sera from recipients were diluted and mixed with various quantities of hen egg
23 lysozyme (1µg/ml – 5pg/ml) or duck egg lysozyme (0.125mg/ml – 0.1µg/ml) and incubated at 4°C

1 overnight. The titre of free anti-IgM^a serum antibodies in solution was assayed by ELISA on plates
2 (Immulon-4) coated with either 20µg/ml DEL or HEL diluted in PBS. Detection was achieved using
3 biotinylated anti-mouse IgM^a (PharMingen) followed by extravidin peroxidase conjugate (Sigma).

4

5 *Microdissection, PCR amplification and cloning of the HyHEL-10 light and heavy chain V*
6 *transgenes*

7 Germinal centres stained with the anti-allotype antibody IgM^a and PNA (Vector) were excised by
8 microdissection under Scott's tap water substitute (0.35% NaHCO₃, 0.2% MgSO₄) using a Nikon
9 Narishige micromanipulator. Photographs were taken with a Nikon Diaphot inverted microscope
10 and a CCD camera. The tissue was digested in 30µl of proteinase K (1.43mg/ml; Boehringer
11 Mannheim) for 1 hour at 50°C followed by heat-inactivation at 95°C for 10 minutes.

12 The HyHEL-10 heavy and light V(D)J transgenes were amplified by two rounds of
13 polymerase chain reaction (PCR) using Expand™ DNA polymerase (Boehringer Mannheim) and
14 nested oligonucleotide primers (Table I). To avoid contamination of reagents with amplified Ig V
15 genes, all procedures before PCR took place in a separate laboratory from that where PCR
16 amplification and all subsequent DNA manipulations were performed.

17 HyHEL-10 V κ and V H genes contain 2 and 13 nucleotide differences from the closest
18 germline genes, V κ 23-43 and V H 36-60, respectively³⁴. The anti-lysozyme HyHEL-10 Ig heavy
19 (H) chain gene construct consists of the LVDJ_H gene segment, μ -switch and constant regions, μ -
20 membrane exons, the δ -constant, secretory and membrane gene segments. The anti-lysozyme
21 HyHEL-10 Ig light (L) chain gene construct comprises the LVJ κ gene segment and the κ -constant
22 region. Both transgene constructs have been described in detail previously²³. External 5'
23 oligonucleotides (LL/LH) hybridised to relatively conserved motifs within the immunoglobulin

1 signal sequences of the VH3 or V κ 23 gene families³⁵. The external 3' oligonucleotides (IH/IL) were
2 specific for the intronic sequences immediately downstream from the JH3³⁶ or J κ 2³⁷ gene
3 segments. Internal oligonucleotides (VL/JL, VH/JH) were complementary to the 5' or 3' ends of the
4 rearranged HyHEL10 light and heavy chain V regions, respectively^{34,38}.

5 The HyHEL-10 light chain V-gene was amplified using the external oligonucleotides LL and
6 IL followed by internal primers VL and JL. The HyHEL-10 heavy chain V- gene segment was
7 amplified with external oligonucleotides LH and IH followed by internal primers VH and JH.

8 The cycling parameters for the PCRs were: 95°C - 2min; (94°C- 1min, 55°C-1min, 72°C-
9 2min) x 40; 72°C-15min except the annealing temperature was 52°C for the primary amplification
10 of the HyHEL-10 light chain V- gene. A wax-gem (Perkin-Elmer) facilitated hot start was used in all
11 PCRs.

12 Ten microlitres of the digested tissue was used in all primary amplification PCRs and 2 μ l of
13 the primary PCR product was used in the second amplification step. Negative control samples
14 containing water instead of DNA were always amplified in parallel with the DNA from the digested
15 tissue. PCR products were purified using either a Qiaquick gel extraction kit or a PCR purification
16 kit (Qiagen), ligated into plasmid vector pCR2 and cloned using the TA cloning system (Invitrogen).

17

18 *Table I approximate position.*

19

20 *Sequencing and analysis of the HyHEL-10 light and heavy chain V region transgenes*

21 Plasmid DNA was isolated from single clones and positive transformants were chosen at random
22 for automated DNA sequencing (DNASHEF, Edinburgh). Sequence chromatographs were analysed
23 with Editview and alignments were carried out by DNAPlot. The MD4 B-cells contain four copies

1 of the transgene, one copy contains a silent mutation at amino acid position 45 of the V κ region ³⁹
2 and was eliminated from any mutational analysis. Dendrograms were produced using sequences
3 with shared subsets of somatic mutations cloned from individual germinal centres; each unique
4 sequence represents the B cell expressing that mutated V-gene.

5

6 **Results**

7 *Kinetics of the DEL-cOVA response*

8 To ensure that DEL coupled to cOVA can elicit B cell help from TCR Tg T cells, as previously
9 reported for HEL, we examined the clonal expansion of Tg T and B cells. HEL-specific Tg B cells
10 and OVA-specific TCR T cells were transferred into naïve congenic (BALB/c-Igh^b x C57BL/6) F₁
11 mice, which were then immunized with either HEL-cOVA or DEL-cOVA in CFA. The lymph
12 nodes were removed from the recipients at days 3, 5, 7, 9 and 14 after immunization, and cells from
13 half the lymph nodes were analysed by flow cytometry. The remaining lymph nodes were used for
14 immunohistochemical analysis. Clonal expansion of both the Tg D011.10 T and MD4 B cells in
15 response to the lower affinity conjugate, DEL-cOVA was detected *in vivo*. As shown in our
16 previous studies ^{20, 21}, the peaks of clonal expansion in response to the high affinity HEL-cOVA
17 conjugate were observed on day 3 and 5 for the Tg T and B cells, respectively, (Fig. 1). In contrast,
18 following immunization with DEL-cOVA, the expansion of Tg OVA-specific T cells was slower
19 than observed in response to the higher affinity, HEL-cOVA. This suggests that B-cells present
20 DEL-acquired cOVA less efficiently to T-cells at the earlier stages of GC formation due to their
21 lower affinity for DEL-cOVA ⁴⁰, or alternatively, that the two antigens are presented by
22 phenotypically distinct antigen presenting cells. There was no significant delay in the B-cell
23 response to DEL-cOVA compared to HEL-cOVA. This observation is in agreement with a recent

1 finding that high and low affinity B-cells have the same intrinsic capacity to respond to antigen⁴¹.
2 These results demonstrate that the MD4 B cells respond to DEL while receiving help from the
3 D011.10 T-cells.

4
5 *Localisation of Tg MD4 B cells and D011.10 T cells during the DEL-cOVA response*

6 Following immunisation, antigen-specific T cells undergo clonal expansion and migrate into B-cell
7 follicles, where they provide help to B cells^{20, 21}. Similar follicular migration for both the Tg B and
8 T cells was displayed in both the DEL-cOVA and HEL-cOVA immunized recipients.
9 PNA-positive GCs containing transgenic IgM^{a+} B-cells were observed at days 5 to 14 with both
10 the high (HEL) and low (DEL) affinity antigens by immunohistochemistry (Fig. 2) and three colour
11 flow cytometry (unpublished data). Although numerous anti-HEL-specific Tg B cells were detected
12 (Fig. 2a, b) within the GCs of both HEL-cOVA and DEL-cOVA immunized mice, no DEL-binding
13 B-cells were detected in the GCs of the DEL-immunized mice (Fig. 2c). The inability to detect
14 DEL-binding B-cells may be due to the sensitivity of the immunochemical system employed. We
15 have established a system in which affinity maturation of a trackable population of antigen-specific
16 B-cells can be analysed during the germinal centre reaction *in vivo*.

17

1 *Somatic hypermutation in germinal centres responding to DEL and HEL*

2 The V κ and VH region transgenes were amplified by nested PCR from 12 IgM⁺, PNA⁺ GCs at
3 various stages of the primary response to both HEL-cOVA and DEL-cOVA. The GCs were
4 identified by PNA staining and consisted of approximately 400-600 cells. Care was taken to avoid
5 the inclusion of PNA negative/non-transgenic B-cells in the surrounding area.

6 The average mutation rate in the germinal centres for both the HEL-cOVA and DEL-cOVA
7 immunized mice was fairly similar ranging from 0.3-0.5% (Table II).

8 *Table II's approximate position.*

9 There was extensive variability in the mutation rate between germinal centres with the
10 occasional GC displaying no mutations within the sample of transgenes analysed. This supports
11 previous findings that substantial clonal expansion within germinal centres can occur without
12 activation of the hypermutation mechanism^{42, 43}, although other reports have suggested that
13 ongoing mutation may occur in all GCs^{44, 45}. Inactivation of the hypermutation machinery may be
14 advantageous at the early stages of germinal centre formation since the accumulation of deleterious
15 mutations within the GC founder cells would be detrimental.

16 Intrinsic mutational hotspots encoded by an RGYW motif [R=A or G, Y = C or T, W = A
17 or T]^{8, 14} were observed within the VH and V κ region transgenes of GCs from HEL-cOVA and
18 DEL-cOVA immunized recipients (Fig. 3). Mutations at these hotspots tend to produce
19 conservative coding changes such as serine to asparagine/threonine, hence preserving antibody
20 structure. Additionally, the somatic hypermutation machinery displays a strong bias of transitions
21 over transversions [59% compared with the 33% anticipated on a random bias⁸]; 60% of the
22 mutations were transitions in this experimental system. These results indicate that somatic

1 hypermutation of the antigen-specific B-cells was occurring in a primary immune response to a
2 protein antigen *in vivo*.

3

4 *Clonal proliferation within GCs*

5 Reconstruction of the hypermutation and clonal expansion events that took place in each GC was
6 undertaken using a previously established procedure for building genealogical dendrograms [Fig. 4⁴²,
7 ⁴⁴].

8 Potentially affinity-enhancing mutations were retained through a number of cell divisions in
9 some B-cell clones (Fig. 4a,b). This may reflect a situation where B-cells with antigen-enhancing
10 mutations are being selected and recirculating for additional rounds of somatic mutation (branching
11 dendrograms). Many GCs at days 9 and 14 from the HEL-cOVA and DEL-cOVA immunised
12 recipients displayed mutationally distinct B-cells emanating from the unmutated transgenes. These
13 dendrograms are described as simple (Fig. 4c) where a number of B-cells containing one or two
14 mutations but no shared mutations were observed. These simple dendrograms suggest that there is
15 an ongoing emigration of B-cells leaving the GC without further rounds of division and mutation.
16 The fact that the transgenic B-cells already have moderate affinity for DEL and high affinity for
17 HEL may explain this observation.

18 Neighbouring GCs from the same section of lymph node were also examined in two cases
19 and no sequences with shared mutations were observed (unpublished data). This is in agreement
20 with previous work suggesting that little or no inter GC B-cell migration takes place and that
21 somatic hypermutation occurs completely independently in individual GCs^{45,46}.

22

23

24

1 *Affinity maturation towards DEL*

2 Changes in the relative affinity of the HyHEL-10 antibody for DEL and HEL were measured by
3 competitive inhibition. Sera from adoptively transferred recipients immunised with either HEL-
4 cOVA or DEL-cOVA were analysed at days 9 and 14. HyHEL-10 antibodies in the sera of DEL-
5 immunized mice consistently showed increased affinity for DEL, both in solution (Fig. 5a) and in
6 solid phase (Fig. 5b), compared with sera from HEL-immunized mice. A unique population of
7 DEL-specific Tg antibodies which could not be inhibited at high concentrations of HEL, was
8 detected in the sera of DEL-cOVA immunised mice (Fig. 5b). These results provide evidence that
9 somatic hypermutation within the V κ and V H region transgenes resulted in increasing affinity for
10 DEL in response to immunisation with DEL-cOVA.

11

12 *Protein modelling of the affinity maturation process*

13 *a) Differences between HEL and DEL*

14 High resolution structural characterisation of the HyHEL-10/HEL interaction ²⁷⁻²⁹ has
15 allowed the examination of individual replacement mutations that have occurred within this
16 experimental system. Crystallography studies have identified 14 hydrogen bonds, 111 Van der
17 Waals contacts and one salt bridge within the interface of the HyHEL-10/HEL complex ²⁸. The
18 shape of the antigen-binding site is a very shallow concavity consisting of acidic and non-
19 hydrophobic residues, bordered by hydrophobic segments ²⁷. There are 21 differences between the
20 amino acid sequence of HEL and DEL [at positions 3, 4, **15**, 33, 34, 37, 57, 68, **73**, **75**, **77**, 82, 85,
21 90, 91, 92, **93**, **97**, 116, 121, 122; 22 in *Duck form III* ²⁵] and these amino acids changes were
22 modelled using the SwissPdb viewer version 3.3b3.

23

1 *b) Differences between HEL and DEL at the HyHEL-10 antigen binding site*

2 Of the 21 differences between HEL and DEL, 6 (in bold, above) lie at the antigen-antibody
3 interface, by inference from the HyHEL-10/HEL structure. All 6 occur in one half of the antibody
4 interface: CDR2(L), CDR1(H) plus CDR3(H) and not CDR2(H), CDR1(L) or CDR3(L). This is
5 illustrated in Figure 6g.

6 This leads to an enquiry as to whether any of these 6 residues are near to any of the
7 antibody residues that mutate during the affinity maturation process *in vivo*. The answer is one of
8 them, viz. residue 93. In HEL, residue 93 is an asparagine whose side chain is specifically hydrogen-
9 bonded to the side chain of the 53V κ glutamine, as seen in Figure 6a. In DEL, residue 93 is an
10 arginine, with a side chain of different hydrogen bond specificity.

11 Nearby, a striking feature of the antigen-binding site in the HyHEL-10/HEL complex is the
12 49V κ lysine, which is prominently exposed and accessible at the centre²⁷. This residue was seen to
13 mutate independently in GC B-cells to an asparagine or a methionine at days 9 and 14, respectively
14 (Fig. 5a). The functional importance of these somatic mutations at residue 49V κ was indicated by
15 modelling of the HyHEL-10/DEL interface (Fig. 6b, 6c). The asparagine produced by point
16 mutation at residue 49V κ interacts directly, via hydrogen-bonding, with the DEL-specific amino
17 acid, arginine 93 mentioned above. The methionine at 49V κ may also result in enhanced binding to
18 DEL through donation of electrons by the sulphur of the methionine to the guanidino group of
19 arginine 93 (Fig. 6c). The importance of this position for antigen binding has been demonstrated by
20 site-directed mutagenesis studies in which conversion of the lysine at residue 49V κ to a threonine
21 increased the affinity of the HyHEL-10 antibody for duck egg lysozyme approximately five-fold²⁵.
22 It is remarkable that the predicted importance of mutations at this position has been confirmed in an
23 antigen-specific B-cell response *in vivo*.

1 *c) Other light chain mutations*

2 At the rest of the antibody-antigen interface where residue changes between HEL and DEL
3 are not seen, some κ chain mutations were found in GC B cells. The serine to asparagine
4 replacement mutation at position 93V κ in CDR3 (Fig. 3a), also previously examined by site-
5 directed mutagenesis, does not significantly alter affinity for DEL, at least in the presence of
6 threonine at residue 49V κ ²⁵. Protein modelling of this mutation, which is next to a contact residue,
7 displays no alteration in hydrogen bonding within the Ag/Ab interface. Thr/Asn/Asp/Ser amino
8 acids often occur in similar hydrogen bonding arrangements and, in such situations, can be
9 considered interconvertible^{47, 48}.

10 Serine 91V κ of the light chain, which is a contact residue in CDR3 with the HEL and DEL
11 amino acid tyrosine 20, is believed to be a mutational hotspot. Conserved coding changes from
12 serine 91V κ to asparagine or threonine were observed at days 5, 9 and 14 in DEL-cOVA responding
13 GCs only (Fig. 3a) and modelling suggests the possibility of maintenance of interactions with
14 Tyr20. The V κ transgenes from DEL-cOVA immunized mice showed a very high preference for
15 transition replacements, particularly in CDR3, which may reflect the requirement for modification
16 to increase interactions with the lower affinity antigen, DEL. This is surprising since most of the
17 non-identical DEL residues at the Ag-Ab interface are interacting with CDR2(L) and HEL/DEL
18 residues close to CDR3(L) are identical.

19 Mutations in the framework regions, notably at serine residues 74V κ and 77V κ in the light
20 chain of monoclonal antibody, HyHEL-8, were also observed in this experimental system. These
21 are considered to be intrinsic hotspots within the light chain nucleotide sequence with conservative
22 coding changes that preserve the antibody structure and are unlikely to affect antigen/antibody
23 interactions.

1 *d) Heavy chain mutations*

2 Overall, the variable heavy chain transgene displayed a lower level of mutation compared
3 with the light chain, with no mutations shared between independent GCs from the HEL-cOVA and
4 DEL-cOVA GCs (Fig. 3b). Protein modelling of the mutation of the CDR2 contact residue serine
5 56VH to asparagine, which was observed at day 9 in a DEL-cOVA GC, showed an enhanced
6 hydrogen-bond interaction with the lysozyme amino acid, aspartic acid 101, which is present in
7 both the HEL and DEL. This mutation also occurs within the HyHEL-8 antibody, which displays
8 higher affinity for DEL ²⁵.

9 Protein modelling of the other coding changes within and around the CDRs typically
10 displayed conserved mutations, which are unlikely to have a major impact on antigen/antibody
11 interaction. Since no recurring mutations were observed we cannot rule out the possibility that some
12 of these individual mutations may have resulted from PCR error.

13 Significantly, no coding changes were detected at the heavy chain residue, aspartic acid
14 32VH that is essential for maintaining the salt bridge to lysine/arginine 97 at the HEL and DEL
15 interface, respectively. Additionally, little mutation was observed in the lower portion of the
16 antigen binding site that is dominated by a cluster of tyrosine residues over the L3 and H2 areas.
17 These Tyr amino acids are believed to create more favourable Ag-Ab interactions through their
18 clustering effectively preventing contact of any other surrounding residues from direct interaction
19 with HEL ^{27, 49}.

20 *Comparison of patterns of somatic mutation between HEL and DEL-cOVA immunized mice*

21 Comparison of the total sequence data from GCs of mice immunised with DEL-cOVA and HEL-
22 cOVA revealed different patterns of replacement mutations. Coding changes such as the mutations
23 at 49Vκ, which increase binding to DEL, were not detected in GCs responding to HEL. Since most

1 of the unshared DEL residues were in the vicinity of CDR2(L), CDR3(H) and CDR1(H), one might
2 predict a larger number of affinity enhancing mutations in that half of the antibody interface. This
3 was not observed possibly because any bias would be small due to sample size.

4 Three light chain mutations were repeated in distinct GCs responding to HEL and DEL. It is
5 unlikely that these mutations could have occurred at random and therefore are likely to have been
6 selected during the GC response. Protein modelling indicated that two of the residues at these sites,
7 Ile29 and Gln90, are essential for the conservation of the structure of the canonical loops ^{50, 51}.
8 According to the canonical class alignment definitions, residue 29 is allowed to be a Val or a Ile, to
9 maintain the class 2 L1 loop and the amino acid at position 90 must be either a Gln, Asn or a His
10 amino acid to preserve the class 1 L3 loop structure ⁵². Modelling of the mutations (IleVκ29→Phe
11 and GlnVκ90→Arg) indicated that they were energetically unfavourable, and were likely to make a
12 structural change in the loop. These canonically important residues were observed to mutate
13 simultaneously with other local amino acids in the same CDR (IleVκ29→Phe with AsnVκ32→Ser,
14 GlnVκ90→Arg with SerVκ93→Ile), suggesting that, the local mutations observed in CDRL1 and
15 CDRL3 could be compensating for the rearrangement of the CDR loop caused by the mutation of
16 these canonical important residues.

17

18 *Endogenous germline genes*

19 Since the primers were specific for the Vκ23 and VH36-60 gene families a few rearranged
20 germline Vκ and VH genes were also cloned from some of the IgM^{a+}, PNA⁺ GCs. More
21 endogenous Vκ23 and VH36-60 genes were cloned from the GCs of DEL-cOVA recipients (35%)
22 than from HEL-cOVA immunised animals (3%), which probably reflects competition between the
23 transgenic and endogenous B-cells for the lower affinity antigen, DEL. The number of mutations in

1 these endogenous V genes was similar to the number of mutations observed in the transgenic light
2 chains. An endogenous VH-gene (with different D and J regions), that was a constituent of a
3 polyreactive anti-histone IgM antibody (Genebank accession no: X63801), was independently
4 cloned at day 9 and 14 from DEL-cOVA immunized mice. This VH region is not a documented
5 germline gene¹¹ and has been previously shown to bind HEL⁵³.

6

7 **Discussion**

8 In this study, we have demonstrated affinity maturation of germinal centre B-cells responding to
9 two defined protein antigens at the cellular and molecular level directly *in vivo* using a novel
10 adoptive transfer model. This *in vivo* system exploits the different affinities of the HyHEL-10
11 antigen receptor for the monomeric protein antigens, hen egg lysozyme ($K_a = 4.5 \times 10^{10}M^{-1}$) and
12 duck egg lysozyme ($K_a = 1.3 \times 10^7M^{-1}$) and the fact that their conjugation to chicken ovalbumin
13 allows provision of T-cell help by cOVA specific D011.10 T-cells^{20, 21}. Previous *in vitro* and *in*
14 *vivo* studies have examined various individual components (clonal expansion, GC formation,
15 affinity maturation) of the antibody response but to our knowledge no sequential detailed analysis
16 of the overall process to a protein antigen has been performed. This system has allowed us to track,
17 in detail, at the level of antigen-specific B cells *in vivo*, all of these molecular and cellular events that
18 occur during the development of an antibody response in one integrated experimental system. Not
19 only will this have implications for a variety of therapeutic approaches but it also provides an ideal
20 system for studying the mechanisms underlying somatic hypermutation *in vivo*.

21 To ensure that DEL coupled to cOVA can elicit B cell help from TCR Tg T cells, as
22 previously reported for HEL, we examined the clonal expansion of Tg T and B cells. Tg T-cells
23 responded more slowly to immunisation with DEL-cOVA than with the higher affinity antigen
24 (HEL-cOVA). This slower T- cell response could be due to the low affinity of the BCR for DEL

1 resulting in less efficient presentation to T-cells and hence slower recruitment of T-cell help ⁴⁰.
2 Alternatively, the lower affinity of the BCR for DEL may lead to competition for antigen between
3 B-cells and other antigen-presenting cells such as dendritic cells for presentation to T-cells.
4 Comparison of the levels of endogenous anti-cOVA IgG1 and IgG2a produced in response to HEL
5 and DEL-cOVA indicates that the latter interpretation may be correct (unpublished data). Previous
6 *in vitro* work has shown that, as the affinity of the BCR increases, there is a corresponding
7 diminution in the amount of soluble antigen required to trigger a response, until the ability to
8 discriminate further increases in affinity disappears above 10^{10}M ⁵⁴⁻⁵⁶. Our study demonstrates for
9 the first time that discrimination between a moderate ($K_a = 10^7\text{M}^{-1}$) and a high affinity ($K_a = 5 \times$
10 10^{10}M^{-1}) antigen also occurs during antigen presentation *in vivo*.

11 As we demonstrated that T and B cell clonal expansion occurs with HEL-cOVA and DEL-
12 cOVA we were next able to employ the system to examine somatic hypermutation. To date,
13 somatic hypermutation has only been investigated in GCs responding to haptens, due to the
14 complexity and heterogeneity of the immune response to protein antigens. Many more contacts
15 occur at the Ab-protein interface, which covers a much larger area, hence the process of antigenic-
16 selection may be very different during the response to a protein antigen compared with the anti-
17 hapten response.

18 The overall mutation rate within the GCs of this adoptive transfer system was fairly low
19 (0.4%) but was significantly higher than the PCR error rate [0.08%;⁵⁷] and comparable with
20 mutation rates from other primary responses ^{45, 58}. Characteristics associated with somatic
21 hypermutation such as a bias towards transitions and intrinsic hotspots, were also observed in the
22 B-cell transgenes. These results confirm the findings of other workers ³⁹ who have also shown

1 somatic hypermutation in the HyHEL-10 V κ transgene in Peyer's patches of intact MD4 mice.
2 However, in this case the antigen driving the response was unclear.

3 Possible reasons why the mutation rate is low in the system we describe are numerous.
4 Firstly, the heavy and light chain transgene cassettes are present in multiple copies and co-
5 integrated into the genome, but not at the endogenous Igh and Ig κ loci, in the MD4 transgenic
6 mouse strain ²³. Each Tg cassette contains a strong Ig promoter and the intronic enhancer, which are
7 essential elements for the initiation of somatic hypermutation ⁵⁹⁻⁶¹. This study and previous work
8 verify that the correct placement of the transgenes within the Ig locus is not critical for mutation ³⁹,
9 ⁶²⁻⁶⁴. However, the Ig heavy chain 3' enhancer downstream from the C μ region is not present. Prior
10 work has indicated that the lack of a 3' heavy chain enhancer reduces the rate of somatic
11 hypermutation ^{65, 66}, which could explain the lower mutation rate observed in the heavy chain
12 transgene compared with the κ transgene in which the 3' enhancer is present. Additionally, the V κ
13 and VH HyHEL-10 transgenes contain 2 and 13 nucleotide differences from the nearest germline
14 genes, V κ 23 and VH36-60, respectively ³⁴. Some of these are in mutational hotspots, which are
15 therefore no longer available for targeting. Finally, the HyHEL-10 BCR displays a moderate affinity
16 for DEL, hence the low accumulation of mutations could be explained by ongoing emigration of Tg
17 B-cells without the requirement for recycling, since they can receive adequate survival signals
18 through the BCR. Some GCs contain many B-cells that have acquired one or two unique mutations
19 only, indicating that they do not contain numerous recycling B-cells. Additionally, other
20 experimental systems may be observing memory B-cells, recycling through germinal centres for
21 further rounds of mutation. The transgenic B-cells are unable to develop into memory cells due to
22 the absence of switch recombination sites ⁶⁷.

1 Having demonstrated somatic hypermutation, we next determined whether the changes
2 observed would be predicted to lead to an increase in affinity for the antigens in question.
3 Competitive inhibition studies demonstrated an increase in affinity of serum antibodies, produced
4 by transgenic B-cells, towards DEL in DEL-cOVA immunized mice. Strong evidence for selection
5 of mutations that shift the affinity from HEL to DEL is provided by the systematic detection of the
6 light chain framework region mutations, 49Vκ (Lysine → Methionine/Asparagine) in different
7 DEL-cOVA recipients. The CDRs of HyHEL-10 are very short and 49Vκ is adjacent to the
8 CDRL2, such that lysine 49 is in the centre of the DEL/HyHEL-10 interface. Protein modelling and
9 previous site-directed mutagenesis experiments demonstrate that these mutations enhance affinity
10 for DEL. Light chain mutations, Lys49Vκ→Thr and Ser93Vκ→Asn, were targeted by site-directed
11 mutagenesis²⁵ due to the observation that HyHEL-10 and HyHEL-8 monoclonal antibodies, which
12 share the same heavy and light chain V-genes (Vκ23, VH36-60), differed in their ability to bind
13 lysozymes from several species of birds, due to the positions of their somatically mutated amino
14 acid residues⁶⁸. Four of the five somatically mutated amino acid residues which differ between the
15 HH8 and HH10 light chains [Vκ residues 49, 74, 77, 93;³⁴] were present within DEL-cOVA GC B-
16 cells and these have been shown to increase affinity for DEL seven fold by chain recombination
17 experiments²⁵. The intrinsic Vκ mutational hotspots Ser74 and 77 may have a subtle effect on
18 affinity although they are distant from the antigen-binding site. Although protein modelling can
19 suggest local changes in antibody structure, it is unable to predict co-operative alterations in
20 structure between somatically mutated residues or long range effects^{69, 70}. Protein modelling of
21 numerous other replacement mutations in the light chain did not reveal direct interactions with DEL.
22 Most coding changes appeared to be neutral, conserving antibody-antigen interactions and antibody

1 structure. Mutations that are neutral in terms of affinity may offer selective advantages towards
2 secretion, folding and physical stability ⁷¹.

3 The observation that the canonically important light chain residues, Ile29 and Gln90 ^{50, 51},
4 were independently mutated to Phe and Arg, respectively, in the HEL-cOVA and DEL-cOVA
5 suggests the influence of selective pressure, although we do not have direct evidence for an effect
6 on antigen binding.

7 Replacement mutations in GC B-cells responding to HEL-cOVA did not appear to be
8 selected since none were repeated independently. The affinity of HyHEL-10 for HEL ($4.5 \times 10^{10} \text{M}^{-1}$)
9 ¹) is believed to be near the effective ceiling for antibodies since B-cells expressing very high affinity
10 receptors do not show a significant advantage over this threshold as measured by competition for
11 antigen bound to FDCs ⁷² or improved antigen presentation to T-cells ⁵⁴. Roost *et al.* ⁷³
12 demonstrated that germline genes encoding antibodies with high affinity for vesicular stomatitis
13 virus showed no affinity maturation over the course of the immune response. Recently, it has been
14 shown that antibody fragments with femtomolar affinities can be evolved by mutagenesis *in vitro*,
15 demonstrating that limitations imposed by the antibody architecture are not intrinsically
16 responsible for the affinity ceiling ⁷⁴. Importantly, our data show that B-cells expressing antigen-
17 receptors close to their affinity threshold are capable of revising their specificity by somatic
18 hypermutation and selection in response to a low-affinity, cross-reactive antigen. This capability
19 would allow further repertoire development of high affinity memory B-cells, which could be
20 important during the immune response to a pathogen undergoing antigenic drift. However, this
21 advantage is offset by the danger that a similar repertoire shift could result in switching from an
22 anti-pathogen response towards a low-affinity, cross-reactive autoantigen (antigen mimicry).
23 Remarkably, mutations in this *in vivo* system recur independently at identical residues indicating

1 that the affinity maturation process within the immune response is extremely selective. The effects
2 of individual point mutations on the affinity of the transgenic HyHEL-10 antibody towards HEL
3 and DEL are currently being investigated using scFv antibody fragments and surface plasmon
4 resonance.

5 To our knowledge, this is the first time that affinity maturation of the GC response to high and low
6 affinity cross-reactive protein antigens has been completely followed through from the clonal
7 expansion of B and T cells in germinal centres to the effects of somatic mutations on the interaction
8 of the antigen-binding site with its epitope. This model also provides an ideal system for studying
9 the mechanisms underlying somatic hypermutation *in vivo*.

10

11

12 **Acknowledgements**

13 We should like to thank Mrs. K. Smith and Dr. G. Sims for their help in training C.A. in the
14 adoptive transfer and microdissection techniques, respectively. This work was supported by the
15 Leverhulme Trust.

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20 **Figure Legends**

21 **Figure 1.** Kinetics of D011.10 T- and MD4 B-cell clonal expansion after immunisation.

22 Cell suspensions containing $1-5 \times 10^6$ CD4⁺KJ1.26⁺ T cells and B220⁺ HEL⁺ B cells were
23 transferred into BALB/c-Igh^b x C57BL/6 F₁ mice. On day 0, transferred recipients were injected s.c.
24 with either 130µg of HEL-cOVA or DEL-cOVA in CFA. The percentage of **A)** D011.10

1 (CD4⁺KJ1.26⁺) T cells and **B**) MD4 (B220⁺IgMa⁺) B-cells in the lymph nodes of adoptively
 2 transferred recipients were determined by flow cytometry at days 3, 5, 7, 9 and 14 after
 3 immunisation. Each time point represents the mean \pm range for at least two mice per group.
 4 Unimmunized controls from each time point were averaged and represented as day 0.

5
 6 **Figure 2.** Immunohistochemical analysis of GCs in lymph nodes of HEL-cOVA and DEL-cOVA
 7 immunised mice.

8 The consecutive sections were stained with either anti-Thy1.2 (brown) or peanut agglutinin (PNA)
 9 and either anti-IgM^a, HEL or DEL (blue). Magnification was at x100.

10 A) Germinal centre from a HEL-cOVA immunised mouse, d9, IgM^{a+} Thy1.2⁺ (left) and
 11 HEL⁺PNA⁺ (right).

12 B) Germinal centre from a DEL-cOVA immunised mouse, d9, IgM^{a+}Thy1.2⁺ (left) and HEL⁺PNA⁺
 13 (right).

14 C) Germinal centre from a DEL-cOVA immunised mouse, d9, DEL⁻ PNA⁺

15

16 **Figure 3.** Representative examples of the V κ and V H sequence data obtained from GCs at various
 17 stages of the HEL-cOVA/DEL-cOVA response.

18 Each sequence is compared to the relevant HyHEL-10 transgene,: **(A)** V κ ; **(B)** V H . Sequences of
 19 CDR1/CDR2 and CDR3 are completely shown as well as the flanking sections where somatic
 20 mutation was observed. Identity with the transgene is indicated by a dash and nucleotide differences
 21 are shown. The number of days after immunization and the antigen used are indicated in the left
 22 hand column. Numbers in front of the sequence alignment are arbitrary sequence names and the

1 letter depicts the different isolated germinal centres. The CDRs in this figure are labeled according
 2 to Lavoie *et al.*, 1999³⁴ and the amino acids in bold represent contact residues.

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4 **Figure 4.** Clonal proliferation of B-cells within individual GCs

5 A simplified hypothesis that each V-gene containing a unique set of mutations represents a distinct
 6 GC B-cell was made. Each branch of the dendrogram represents mutations that were shared by
 7 several V-genes, using the assumption that the mutation event only occurred once in a precursor cell
 8 and was inherited by daughter cells. Numbers on branches represent the number of mutations (silent
 9 mutations are enclosed in brackets) between the different sequences. Each circle symbolises a B cell
 10 and letters within the circles depict individual sequences.

11 Branching dendrograms represent VH (**A**) and V κ (**B**) sequences derived from independent GCs
 12 from day 9 and 14, respectively, in the DEL-cOVA response. The VH antigen-selected hotspot
 13 Ser56→Asn is symbolised by B-cells, I, J and K on the right hand side of the dendrogram A. The
 14 ‘affinity enhancing’ mutation Lys49→Met of VL is depicted by letters E, F, G and H on the left
 15 hand side of the dendrogram B, and B-cell, H, also contains the intrinsic hotspots, Ser77→Ile and
 16 Ser91→Asn. (**C**) depicts a simple dendrogram produced from the V κ sequence data from a HEL-
 17 cOVA GC.

18

19 **Figure 5.** Relative affinity of IgM^a transgenic antibodies in sera from HEL-cOVA and DEL-cOVA
 20 immunised recipients at days 9 and 14, by competitive ELISA. **A**) Competitive binding of IgM^a
 21 antibodies to HEL (solid phase) by DEL (soluble phase); **B**) Competitive inhibition of IgM^a
 22 antibodies to DEL (solid phase) by HEL (soluble phase); (average of two identical experiments).

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Figure 6. Protein modelling of antibody – lysozyme interactions.

A) An area of the antigen-binding site showing details of the interactions between HEL and the mouse HyHEL-10 anti-HEL antibody, based on the crystal structure of the Fab-antigen complex (28). **B)** Model of the same region showing the effects of the LysVκ49 → Asn mutation on the interaction between the maturation mutant antibody and DEL. **C)** Model of the same region showing the effects of the LysVκ49 → Met mutation on the interaction with DEL. **D-G** are views of the HyHEL-10 antigen binding site seen perpendicularly to the antibody-antigen interface. **D)** employs the colour scheme of F and shows the position of the interacting side chains of residues LysVκ49 (red) and the HEL Asn93 (superimposed in black). **E)** shows the two V domains: VH in green and Vκ in blue. **F)** is the same as E with the positions of the CDR regions shown by different colours. Vκ: CDR1, pale blue; CDR2, grass green; CDR3, violet. VH: CDR1, white; CDR2, orange; CDR3, yellow. **G)** is the same as F except that the side chains of the six interface residues differing between HEL and DEL are superimposed in black. Their size is reduced in D and G for clarity and the residue number is indicated at the right hand side.

1 **Table I.** Oligonucleotide primers

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Oligo. name	Sequence (5'-3')
LL	TGGAYTYCAGCCTCCAGA
IL	TAACACCTGATCTGAGAATG
VL	GATATTGTGCTAACTCAGTCTCC
JL	TTATTTCCAGCTTGGTCCC
LH	TGTTGACAGYCVTTCCCKGGT
IH	GTTGAATCTTGATTCCC GTT
VH	GTGCAGCTTCAGGAGTCAGGA
JH	TGCAGAGACAGTGACCAGAGT

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1 **Table II.** Summary of sequencing results obtained from individual GCs at different days after
 2 immunisation with DEL-cOVA or HEL-cOVA.

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4 A. *DEL-cOVA* –immunised mice

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GC	Day	V gene	Description	No. of unique sequences	No. of mutations	% unmutated transgenes
B	5	H	Simple	5	6	63.6 (7/11) ^a
D	5	L	Branching	5	7	55.5 (5/9)
		H	Simple	3	2	81.8 (9/11)
A	9	L	Simple	4	5	42.9 (3/7)
H	9	L	Branching	4	7	20 (1/5)
		H	Extensive Branching	9	13	33.3 (3/9)
J	9	H	Simple	4	5	0 (0/7)
L	9	H	No S.M ^b	1	0	100 (6/6)
A	14	L	Extensive Branching	7	14	38.5 (5/13)
		H	No S.M	1	0	100 (11/11)

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7 Average VL mutation rate = 0.49%

8 Average VH mutation rate = 0.32%

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B. HEL-cOVA immunised mice

GC	Day	V chain	Description of B cell clones	No. of unique sequences	No. of Mutations	%unmutated transgene
C	9	L	Branching	11	15	41.2 (7/17) ^a
		H	Branching	9	18	43.8 (7/16)
F	9	L	Simple	4	6	30 (3/10)
G	9	L	Simple	7	6	40 (4/10)
D	14	H	Simple	4	4	70 (7/10)
K	14	L	Simple	4	3	40 (4/10)

Average VL rate = 0.36%

Average VH rate = 0.5%

Results obtained from the analysis of primary GCs over days 5, 9, and 14. Data from individual GCs are shown on separate lines. The number of unique somatic mutations per GC was evaluated by counting mutations shared by two or more VH/VL clones only once. Average mutation frequency per V gene base pair was calculated by dividing total number of somatic mutations observed by the total number of base pairs sequenced. Dendrograms containing a number of mutationally distinct B-cells emanating from the unmutated transgene were described as simple. A branching dendrogram represents a number of B-cells with shared and unique mutations, which can be followed along one arm of the dendrogram.

^a The number of unmutated transgenes in the total number of sequences analysed is shown in parentheses. ^b No S.M = no mutations were observed.

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