International Immunology, Vol 8, No. 7, pp. 1067–1075 © 1996 Oxford University Press

Defects of somatic hypermutation and class switching in alymphoplasia *(aly)* mutant mice

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Keywords: *aly/aly* mice, immune response, somatic hypermutation

Abstract

The alymphoplasia *(aly)* mutation of mice causes the systemic absence of lymph nodes, Peyer's patches and well-defined lymphoid follicles in the spleen. We found that antibody responses are elicited, albeit weakly, to either T cell-dependent or T cell-independent antigen by *aly/aly* mutants. However, isotype switching was defective. The T cell-dependent immune response was not elicited in splenectomized *aly/aly* mice. Neither hypermutation nor germinal center formation was observed in *aly/aly* mice. These results suggest that T–B collaboration requires either lymph nodes or spleen, and that hypermutation and affinity maturation depend on germinal center formation.

Introduction

Antibody production in the spleen and lymph nodes takes place primarily in two distinct multicellular organizations called the antibody-forming cell foci and germinal centers. Foci are aggregates of plasma cells at the periphery of the periarteriolar lymphatic sheath and are the major source of early circulating antibodies Germinal centers are well-defined structures in lymphoid follicles that contain aggregates of B cells (1). B cells in germinal centers are readily identified by their ability to strongly bind a lectin, peanut agglutinin (PNA) (2).

Ig variable region (V) gene diversity is generated by somatic DNA rearrangement in B cell precursors of the bone marrow and fetal liver. During the course of the antigen-specific immune response, V gene diversity is further enhanced by somatic hypermutation in mature B cells of germinal centers (3). As a consequence, B cell clones express Ig with high affinity towards their respective antigens. Such high-affinity clones selectively proliferate and then dominate in the memory cell pool (4). Affinity maturation by Ig hypermutation and selection are thought to occur exclusively in germinal centers, but not in foci (5,6). Germinal center formation is a complex process that requires interactions of B cells with T cells, follicular dendritic cells, antigen–antibody complexes and complements (7,8), but the molecular basis of germinal center formation and Ig hypermutation remains unclear. Germinal centers and foci are also sites for class switching which takes place by antigen stimulation and T cell help during the maturation of the immune response (9). Class switching diversifies the effector function of the antibody, which contributes to the elimination of pathogens. Isotype switching is directed by cytokines such as IL-4, IL-5, IL-10, IFN-γ and transforming growth factor (TGF)-β, in not only T cell-dependent but also T cell-independent type 2 antibody responses (10,11).

The *aly* mutation was originally found as a spontaneous autosomal recessive mutation that causes the systemic absence of lymph nodes and Peyer's patches in C57BL/6 mice (12). Although a very similar phenotype was reported for lymphotoxin-α-deficient mice (13,14), the *aly* locus and the lymphotoxin-α gene are mapped at mouse chromosomes 11 and 17 respectively. The spleen of *aly/aly* mutants is devoid of well-defined lymphoid follicles and its white pulp is atrophic. The level of serum IgM in *aly/aly* mutants is approximately one-third of that found in heterozygotes, while those of serum IgG and IgA are severely depressed (12). *aly/aly* mutants are deficient in both humoral and cell-mediated immune responses, although they have mature T and B cells as determined by their cell surface markers (12,15). The *aly/aly* mutant thus provides a unique system which allows us to

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Transmitting editor: K Takatsu

Received 22 January 1996, *accepted* 27 March 1996

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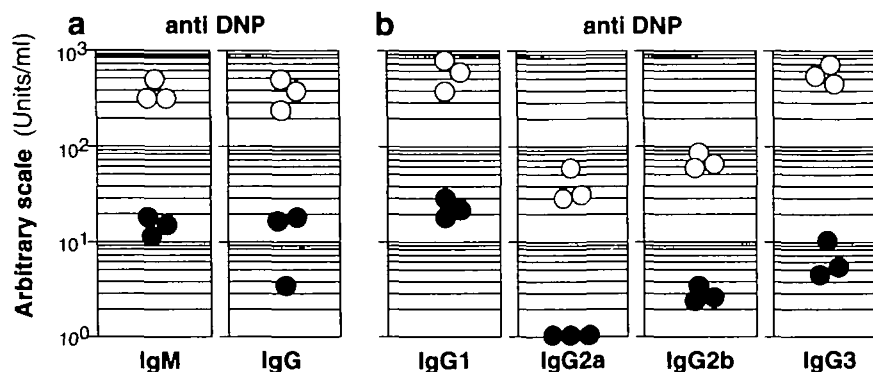

Fig. 1. Immune response of *aly/aly* mice to a T cell-independent type 2 antigen. DNP-specific antibody titers in sera obtained 14 days after an i.p. injection of DNP-Ficoll DNP-specific titers are indicated in relative scale The titer comparison of a given isotype should be done between *aly* homozygote and heterozygote mice, but not among different isotypes Open circles, *aly* heterozygote mice, closed circles, *aly* homozygote mice (a) DNP-specific IgM and IgG (b) DNP-specific antibodies of IgG subclasses

test whether germinal centers and foci in lymph nodes and spleen are absolutely essential for hypermutation and class switching of Ig.

In this study, we report that the *aly/aly* mutant can elicit weak antibody responses to both T cell-dependent and -independent antigens. However, no Ig hypermutations were detected and no germinal centers were generated in the spleen of the *aly/aly* mutant. Furthermore, defective Ig isotype switching took place presumably in foci of the spleen of the *aly/aly* mutant. It thus appears that maturation of the immune response including Ig hypermutation, affinity maturation and Ig isotype switching requires the developed architecture of foci and/or germinal centers in lymph nodes and spleen.

Methods

Mice

All experiments were performed with 20- to 40-week-old *aly* homozygote and heterozygote mice which were maintained under specific pathogen-free conditions.

Immunization

To measure serum antibody titers in T cell-independent responses, mice were immunized i.p. with 10 μg of dinitrophenylbenzene (DNP)-Ficoll in PBS and bled on day 14. To investigate the T cell-dependent response, we immunized mice i.p. with 200 μg of (4-hydroxy-3-nitrophenyl)acetyl-human γ-globulin (NP-HGG) in Freund's complete adjuvant (Nakalai Tesque, Kyoto, Japan). To elicit the secondary and tertiary T-dependent responses, 200 μg of NP-HGG in Freund's complete adjuvant were injected into their peritoneal cavity in 4–6 week intervals. Immunized mice were bled on day 7 after each immunization. Splenectomy was done 1 month before primary immunization and secondary immunization was done as described above. All antigens were kindly provided by Dr T. Kina (Kyoto University)

Titration of serum Ig

Antigen-specific antibodies were detected by ELISA (16). Serum antibody concentrations (U/ml) were determined by comparing titers with standard curves generated by using hapten-non-reactive control mAb of various isotypes (Chemicon International, Temecula, CA).

Immunohistochemistry

To test whether germinal centers are developed in spleens of *aly* homozygotes, NP-immunized *aly* homozygotes and heterozygotes were killed on day 10 after the last boost. Their spleens were removed, fixed and embedded in polyester wax (BDH, Poole, UK) as described (17). Spleens were cut into 5 μm thick sections and mounted onto 0.1% neoprene-coated slides. Tissue sections were stained with hematoxylin & eosin in standard procedures. Sections were stained with biotinylated PNA (EY Laboratories, San Mateo, CA) or biotinylated anti-mouse B220 (RA3-6B2) and visualized using the VECTASTAIN ABC-AP reagent (Vector, Burlingame, CA) and alkaline phosphatase substrate kit I (Vector Red; Vector). The sections stained with PNA were counter-stained with methyl green.

Isolation of splenic λ1-expressing B cells

An *aly/aly* mutant and a control *aly* heterozygote mice were immunized with NP-HGG three times and twice respectively, and killed when their serum titers of NP-specific IgG reached 570 and 32,000 U/ml respectively. Spleen cells were stained with the biotinylated anti-λ1 antibody Ls136 (a gift from Dr T. Takemori, NIH, Japan) and streptavidin–phycoerythrin. $Ls136^+$ cells were 0.4% in mutant cells and 0.6% in control cells, and $\lambda1^+$ cells were enriched to a level of 2.6% from mutant cells and 17.5% from control cells using a magnetic cell sorter (18)

Amplification of rearranged V_H186.2 genes and DNA sequencing

Genomic DNA of the sorted cells was isolated for amplification of rearranged V_H186.2 genes by PCR. Fifty cycles of PCR were performed using the following oligonucleotide primers: V_H186.2, 5′-TTGTCGACAGTTACTGAGCACACAGGACCTC-3′ (annealing to the 5′ portion of V_H186.2) and J_H2, 5′-GGT-CGACGGTGTCCCTAGTCCTTCATGACC-3′ (annealing to the

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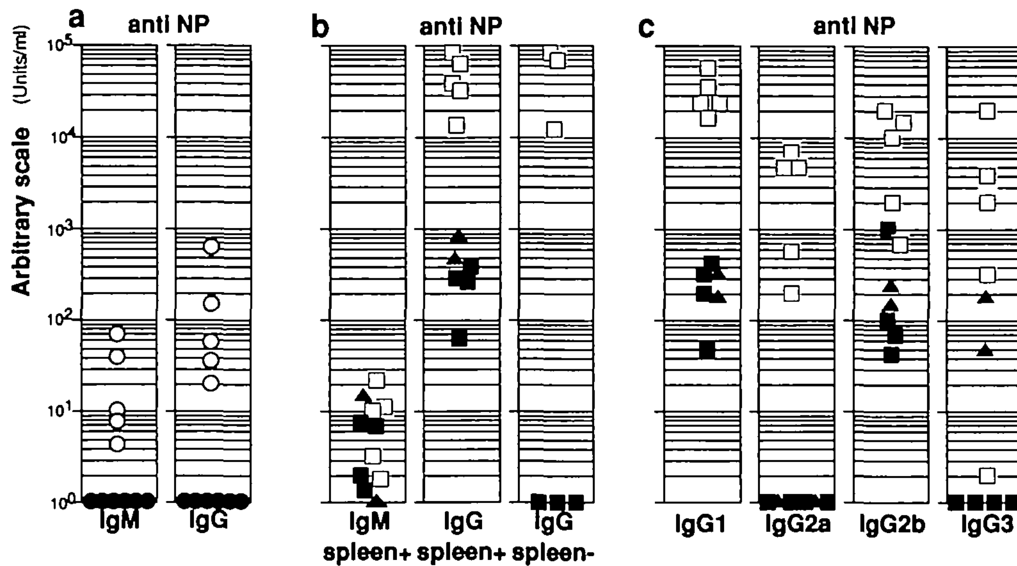

Fig. 2. Immune response of *aly/aly* mice to a T cell-dependent antigen Serum concentrations of NP-specific antibodies of *aly/aly* and heterozygote mice immunized with NP-HGG are indicated in relative scale The titer comparison of a given isotype should be done between *aly* homozygote and heterozygote mice, but not among different isotypes Circles, titers in primary responses; squares, titers in secondary responses; triangles, titers in tertiary responses Open and closed symbols represent heterozygote and homozygote mice respectively. (a) The primary immune responses NP-specific IgM and IgG in sera obtained at day 7 after the first immunization with NP-HGG. (b) Secondary or tertiary responses at day 7 by repeated immunization with NP-HGG. The left column shows NP-specific secondary or tertiary responses of non-splenectomized mice The right column shows the titers in the secondary response seen in *aly* homozygote and heterozygote mice, both of which were splenectomized. (c) Serum titers of NP-specific antibodies of IgG-subclass in secondary or tertiary responses

intron between J_H2 and J_H3 segments). The temperature profile of the amplification was 95°C for 1 min and 72°C for 2.5 min. Amplified DNA fragments were cloned into the pGEM-T vector (Promega, Madison, WI) by standard procedures. The D regions of the NP-specific V_H genes are known to encode three tyrosine residues (19,20). To isolate NP-specific V_H genes, colony DNAs were tested by PCR amplification with the $V_H186.2$ primer and the 5′ DFL16.1 primer, 5′-TAGCTACTACCGTAGTAATA-3′ (annealing to the 5′ part of DFL16.1). The temperature profile of this amplification was 94°C for 1 min, 60°C for 2 min and 72°C for 2 min. Four hundred colonies from the *aly/aly* mutant and 120 colonies from the control mouse were chosen. Direct sequencing of the amplified DNA was carried out with the J_H2 primer (see above) and V_H genes which contain TATTACTAC at the 5′ part of the D segment and the sequence CCCTCC at codon 74–75 of $V_H186.2$ were selected for mutation analyses. The sequence CCCTCC is a hallmark to distinguish $V_H186.2$ (21) from other homologous V_H segments of the J558 family (22).

Results

Response of aly/aly mutants to the T cell-independent type 2 antigen

Since the previous study indicates that *aly/aly* mice have very weak, if any, immune responses to antigens (12), we decided to examine the immune response of *aly/aly* mice quantitatively in more detail. We first measured the antibody response of *aly/aly* mutants to a typical T cell-independent type 2 antigen, DNP-Ficoll (Fig. 1). Anti-DNP IgM and IgG levels in *aly/aly* mutants were elevated after immunization although they were at least one order lower than those in control mice (Fig. 1a). Small amounts of IgG1, IgG2b and IgG3 but not IgG2a antibodies specific to DNP were detected in sera of immunized *aly* homozygotes (Fig. 1b). Thus, *aly/aly* mutants are capable of mounting immune responses against the T cell-independent type 2 antigen although these responses appear to be defective.

Response of aly/aly mutants to the T cell-dependent antigen

We have carried out similar quantitative studies on the immune response of *aly/aly* mice to a T cell-dependent antigen, NP-HGG. In *aly* homozygotes, serum antibodies specific to NP did not increase by the first challenge (Fig. 2a). However, the secondary or tertiary i.p. immunization elevated the serum levels of NP-specific IgM to the control level in ~50% of the *aly* homozygotes. Anti-NP IgG levels in the *aly/aly* mice reached only ~1% of those found in control mice (Fig. 2b). Serum titers of IgG subclasses specific to NP after the secondary or tertiary immunization of the homozygotes are lower than those of control mice but clearly detectable except for IgG2a (Fig. 2c). In the T cell-dependent as well as T cell-independent antibody response, NP-specific IgA could not be detected in either *aly/aly* mice or heterozygote controls (data not shown). However, total IgA antibodies in sera were detected in heterozygote controls, but not in *aly/aly* mice even after the repeated immunizations. In spite of low antibody titers and defective Ig isotype switching, *aly* homozygotes were clearly able to mount T cell-dependent immune responses.

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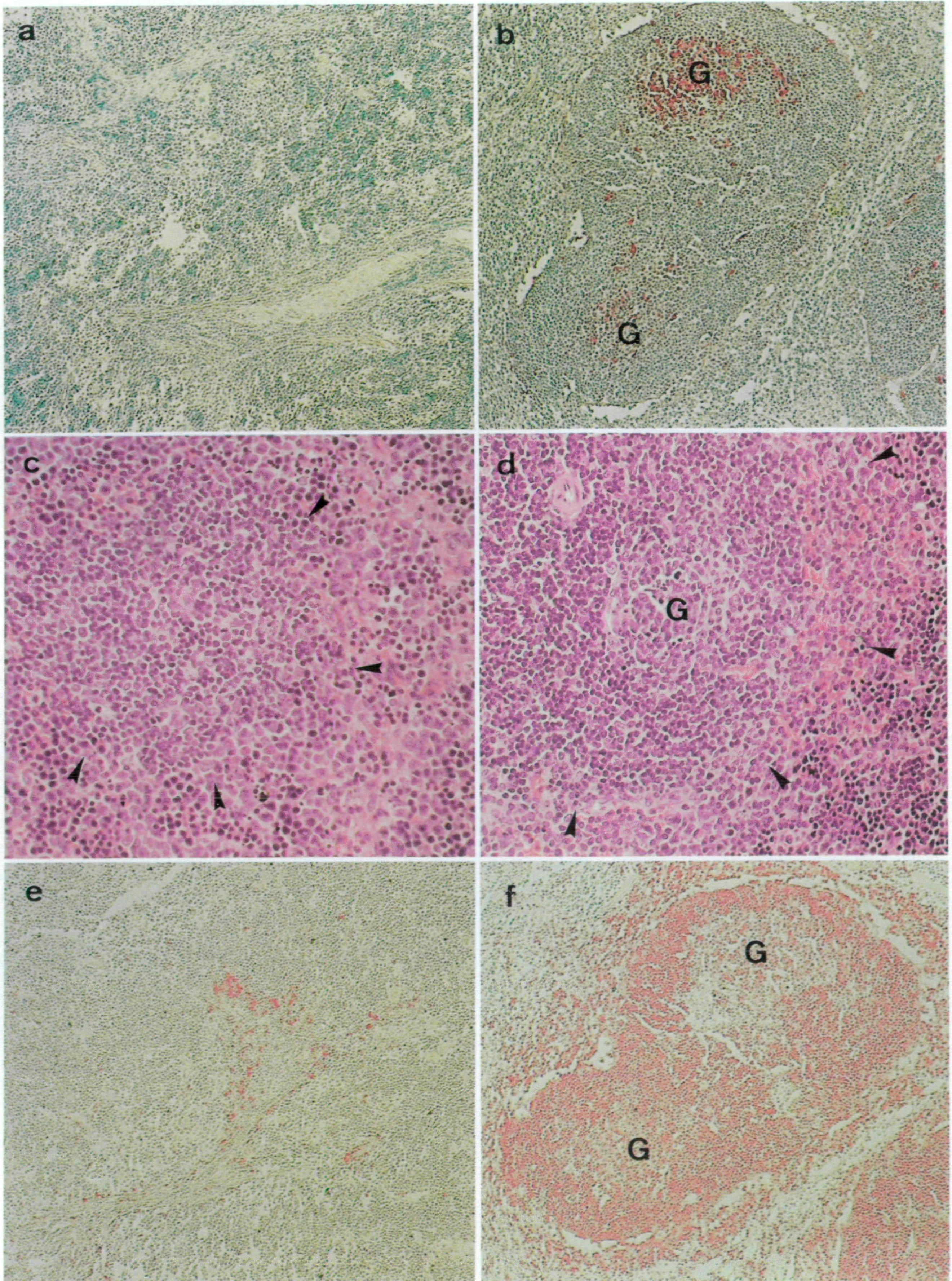

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Spleen is essential for the T cell-dependent antibody response in aly/aly mutants

Since *aly/aly* mice do not contain lymph nodes, we suspected that spleen might be the site for T–B interaction in the T cell-dependent immune response. To test this possibility splenectomized *aly/aly* mice were challenged with NP-HGG. After splenectomy *aly/aly* mice could not produce anti-NP IgM and IgG antibodies even after repeated immunizations (Fig. 2b, right panel), whereas splenectomized heterozygote animals generated the same levels of NP-specific antibodies in sera as non-treated control heterozygote mice. Therefore the spleen is essential for antibody production against the T cell-dependent antigen in *aly/aly* mutants.

Absence of germinal centers in spleens of aly/aly mutants

Since maturation of the immune response such as Ig hypermutation and affinity maturation occurs in germinal centers (5,23), we examined whether PNA^{hi} germinal center B cells were detectable in the spleen of *aly/aly* mutant mice. Immunohistochemical staining of spleen sections revealed that *aly* homozygote animals immunized with NP did not develop germinal centers as shown by the absence of PNA-binding cells (Fig. 3a and b). Hematoxylin & eosin staining of spleen of immunized mice showed that the white pulp of *aly/aly* mice was atrophic and that the major cell population which occupied the spleen was myeloid cells including a large number of erythroblasts, but not lymphocytes (Fig. 3c and d). Anti-B220 staining supported the paucity of B cells in the white pulp of *aly/aly* spleen (Fig. 3e and f) A few B cells can be seen in scattered areas around terminal arterioles in the *aly/aly* spleen. Both lymphoid follicles and marginal zones were obscure in the *aly/aly* spleen, in comparison to the heterozygote spleen. Thus, repeated immunizations could neither develop germinal centers nor normalize the architecture of the white pulp in spleens of *aly* homozygotes.

Antibodies generated in aly/aly mice have broader specificities to antigens

Since somatic Ig hypermutation and affinity maturation are thought to be dependent on the germinal center, we have investigated whether Ig hypermutation and affinity maturation take place in *aly/aly* mutants. For this purpose, we first analyzed the relative antigen-specificities of antibodies generated in *aly/aly* and heterozygote mice. NP-immunized *aly/aly* sera contained antibodies with relatively stronger binding capacities against DNP or trinitrophenylbenzene (TNP) than NP-immunized heterozygote sera (Table 1), indicating that antibodies generated in *aly/aly* mice have broader specificities and that the affinity maturation of antibodies may be defective in *aly/aly* mutants.

Absence of somatic hypermutation in aly/aly mutants

We then examined whether the broader specificity of anti-NP sera is due to the absence of Ig hypermutation in *aly/aly* mutants. The antibody response of IgH^b mice to this hapten has been well characterized and shown to be highly restricted (19,20). It is dominated by λ1-bearing antibodies expressing the V_H186.2 segment in association with the DFL16.1 and J_H2 segments. Approximately 50% of these antibodies contain the 5′ part of the germline DFL16.1 sequence encoding three consecutive tyrosine residues. We analyzed VDJ sequences of genomic DNA recovered from magnetic-cell-sorted $\lambda1^+$ splenic cells of an *aly/aly* mutant and a heterozygote whose sera showed typical hapten specificities after hyperimmunization with NP (Table 1). Using a PCR cloning approach, we selected the NP-specific V_H genes and determined their nucleotide sequences (Table 2). Ten rearranged V_H genes from the *aly* homozygote and 13 from the heterozygote were used to determine their complete sequences. Among those, we found five and seven independent NP-

Table 1. Comparison of specificity of serum IgG antibodies generated in *aly/aly* mutants and heterozygotes[a]

Hapten[b]	IgG ratio[c]	
	aly/aly (*n* = 5)	*aly/*+ (*n* = 6)
NP	2 ± 0 (1)	100 ± 53 (74)
DNP	5 ± 7 (3)	100 ± 55 (130)
TNP	5 ± 5 (6)	100 ± 71 (141)

[a]All mice were immunized i p. with 200 µg of NP-HGG more than twice as described in Methods

[b]Hapten-specific IgG were detected by ELISA using each hapten-coated microtiter plate The ratio comparison of a given hapten should be done between *aly* homozygotes and heterozygote mice, but not among different haptens

[c]Relative IgG titer binding to each hapten with the average value of the *aly/*+ mice taken as 100 IgG ratios of mice used for mutation analysis are shown in parentheses. Mean ± SD.

Table 2. Strategy for selection of NP-specific V_H genes

	aly/aly	*aly/*+
Screened colonies[a]	4200	3200
Selected with D_H primer[a]	400	120
Clones with the marker sequences[b]	10	13
Clones used for mutation analysis	5	7
Clones not used in this study		
stop codon	1	1
out of frame	1	4
related V_H gene	0	1
sister clones	3	0

[a]Screening and selection methods are described in Methods

[b]CCCTCC at codons 74–75 of V_H and TATTACTAC at the 5′ part of D_H

Fig. 3. Absence of germinal centers in *aly/aly* mouse spleen. *Aly* homozygote and heterozygote mice were immunized i p. with NP three times and twice respectively Spleens were taken at day 10 after the last boost and sections were prepared from spleens of *aly/aly* mice (a, c and e) and heterozygote control mice (b, d and f). Tissue sections were stained with PNA (red) and methyl green (a and b), with hematoxylin & eosin (c and d) or with anti-B220 (red in e and f) Arrows show the white pulp of spleen of each mouse. G, germinal center.

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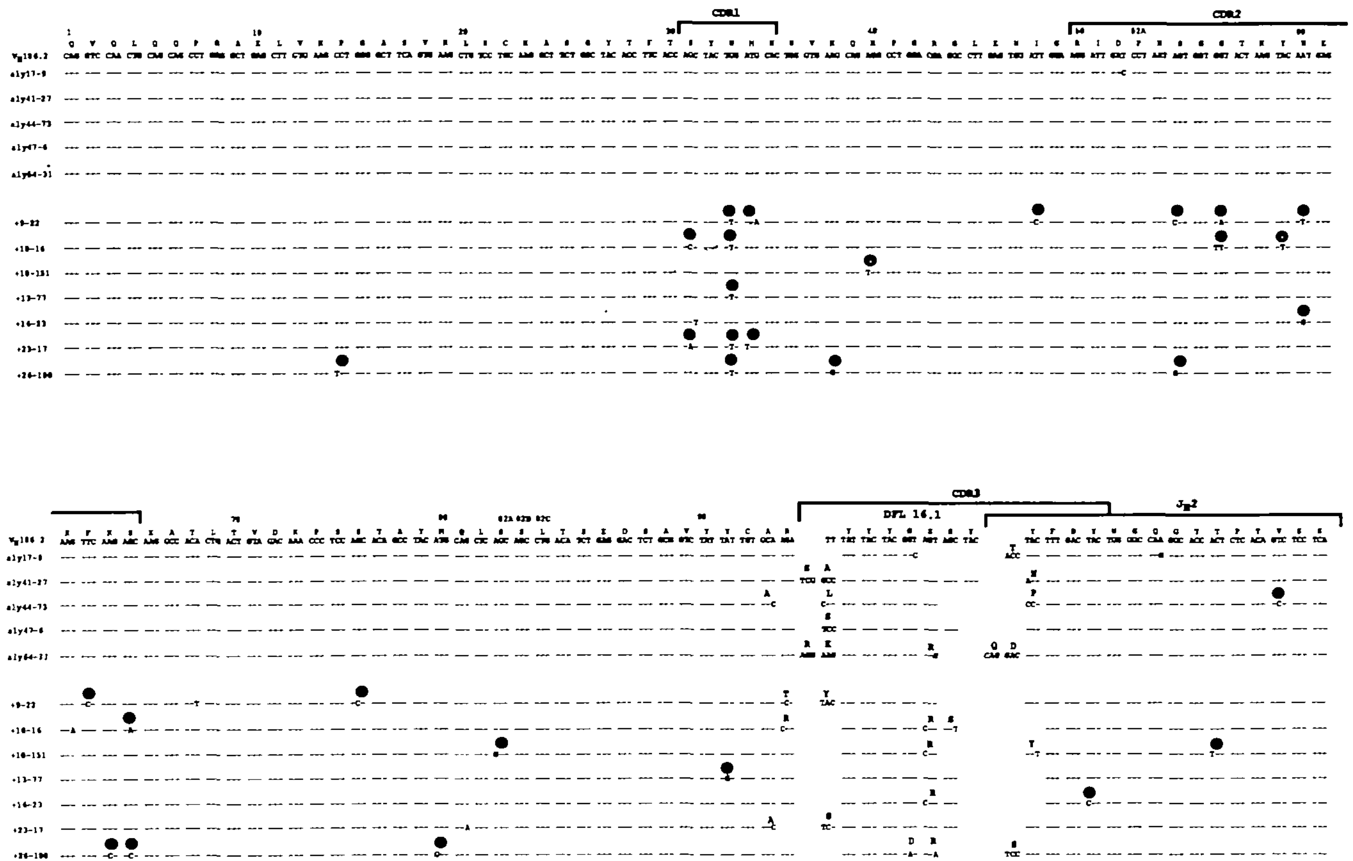

Fig. 4. Sequences of functional NP-specific V_H186 2 genes in the NP-immunized *aly* homozygote and heterozygote mice The upper five sequences designated by 'aly' are derived from an *aly/aly* mouse. The lower seven sequences designated by '+' are from a control mouse The sequences are compared with the germline V_H186.2 segment, the DFL16.1 segment and the J_H2 segment. Nucleotides identical to the germline reference sequences are indicated by dashes Blanks at the 5' end of the D and J_H segments denote the absence of those codons from the sequenced gene. Marked in black circles are replacement mutations relative to the reference sequences The codons are numbered according to (36)

Table 3. *Somatic mutations in rearranged V_H186.2 and J_H2 sequences in aly/aly mutant mice*[a]

Mice	No of nucleotides	CDR1+2 total	R	S	R/S	Framework region total	R	S	R/S	Total mutated bases	Corrected mutated bases[b]	Mutation rate[c]	Corrected mutation rate[b,c]
aly/aly	1686	1	0	1	0	2	1	1	1	3	0.3	1.8×10^{-3}	$1\ 8 \times 10^{-4}$
aly/+	2358	23	21	2	10 5	12	10	2	5	35	31.1	$1\ 5 \times 10^{-2}$	$1\ 3 \times 10^{-2}$

[a]*Nucleotides exchanges in the V_H and J_H segments Nucleotides replacements at the $V_HD_HJ_H$ junctions were not scored as mutations.*
[b]The error rate of the Taq polymerase ($1\ 6 \times 10^{-3}$/bp) was determined in our experimental conditions and deduced
[c]Mutation per base pair.

specific V_H sequences from the homozygote and heterozygote respectively (Fig. 4). The remaining sequences were either identical overlaps or non-functional mutants with frame-shifts or stop codons. One V_H gene from the heterozygote, which contained the V_H145 segment similar but not identical to V_H186.2, was excluded from the analyses (21).

Each functionally rearranged VDJ sequence corresponds to an independent recombination event as its V_H–D–J_H junction is different from each of the others. All sequences shown contain the V_H186.2 segment and three tyrosines at the 5' part of DFL16.1 which is known to be characteristic to V_H sequences involved in the NP response. We observed three mutations in a total of 1686 nucleotides determined for five rearranged genes of the *aly/aly* mutant and 35 mutations in a total of 2358 nucleotides determined for seven genes of the control, corresponding to the mutation frequencies of 1.8×10^{-3}/bp (= 1/562) and 1.5×10^{-2}/bp (= 1/67) respectively. In these experiments, the error rate of the Taq polymerase

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was determined as 1.6×10^{-3}/bp by sequencing known genes. When the Taq error is deducted, the mutation rates are 1.8×10^{-4}/bp in the *aly/aly* mutant and 1.3×10^{-2}/bp in the heterozygote mouse (Table 3). The NP-specific V_H gene mutation frequency of the homozygote (1.8×10^{-3}/bp) is almost the same level as the frequency of the Taq error in the course of PCR. Thus, few mutations, if any, were introduced in the *aly/aly* mutant during repeated immunizations. On the other hand, the mutation frequency of the heterozygote (1.5×10^{-2}/bp) is in good agreement with the mutation rate of 1.9×10^{-2}/bp reported for C57BL/6 mice immunized repeatedly with NP (21,24) and 10-fold higher than the error frequency by the Taq polymerase.

Absence of high-affinity variants in the aly homozygote

Not only the frequency but also the locations of mutations found exhibit striking differences between the *aly* homozygote and the heterozygote control. Although only three mutations were detected in the *aly/aly* V_H region (Table 3), the key mutation conferring high affinity to the NP hapten, a tryptophan to leucine exchange in codon 33, commonly seen in anti-NP antibodies (21,24–26), is absent in the *aly/aly* mutant. By contrast, 70% of the mutated genes that we isolated from the NP-primed control mouse contain this particular amino acid substitution (Fig. 4). These results indicate that affinity maturation of antibodies during the immune response, which is due to the combination of hypermutation in the V gene and antigen-driven clonal selection, is greatly impaired in the absence of germinal centers in the *aly/aly* mouse. However, it is not clear whether or not this defect is due to impaired clonal selection because of the extremely low frequency of hypermutation in the *aly* homozygote.

In addition to the key mutation at codon 33, other amino acid residues were recurrently mutated in the V_H genes from the control mouse (Fig. 4). Within the CDR1 and CDR 2 regions, codons 31, 34, 54, 56, 60 and 65 are mutated more than once. It has been shown that most of these mutations are the result of mutational hot spots and reflect intrinsic properties of the hypermutation mechanism rather than selective forces for hapten binding affinity (20,24,25). These mutational hot spots are not mutated in the *aly/aly* mouse, indicating that the intrinsic hypermutation mechanism itself does not work in the absence of germinal centers.

Discussion

In this study, we showed that *aly/aly* mutants respond, albeit weakly, to both T cell-dependent and T cell-independent type 2 antigens. Splenectomized *aly/aly* mice could not elicit the T cell-dependent immune response, suggesting that either the spleen or lymph nodes are essential for T–B interaction. Defects in the primary immune response are illustrated by reduction of serum titers and incomplete class switching in *aly/aly* mice. In the secondary immune response, histological study showed no germinal center formation in *aly/aly* spleen. In addition, Ig somatic hypermutation and antigen-driven B cell selection are almost completely impaired, indicating that these activities are germinal center dependent.

It has been reported that Ig somatic hypermutation and the subsequent selection of high-affinity variants are events specifically taking place in germinal centers, but not in foci (5,6). Recently, it has been shown that the DNA sequences surrounding the V gene determine the target of hypermutation, even if the V sequence is replaced by heterologous sequences (27). Somatic mutations take place in the PNA^{hi} population of the germinal center, whereas few mutations are seen in the PNA^{low} population which contains foci B cells (5,6). Taken together with our results, the *aly* mutation results in the absence of the PNA^{hi} population which may be causative to defects in hypermutation, affinity maturation of antibody and antigen-driven selection. A recent report that the B cell clones containing the identical VDJ rearrangements are present in both foci and germinal centers suggests the presence of one common precursor for B cells in both sites (23). On the other hand, transfer experiments are in agreement with a view that foci and germinal center B cells are generated from separate precursor subpopulations (28). It remains unclear whether the absence of germinal center B cells is due to the absence of their precursors or the defect in the migrating process of precursor B cells into the germinal center in *aly* homozygotes.

An overall reduction of B cells in mutant mice may in part account for the observed low serum titers after repeated immunizations. On average, *aly* homozygotes have only ~20% of B cell numbers in their spleens as compared with controls (our unpublished data). However, our studies revealed that the amounts of antigen-specific IgG of immunized *aly/aly* mice were 1/50–1/100 of those in control mice. Such reductions may not be solely due to decreased numbers of B cells. The histological data presented here show that there is little evidence for B cell proliferation in either foci or follicles of *aly/aly* spleens, which normally precedes antibody secretion in foci and germinal center formation (Fig. 3). Thus, our results suggest that, upon antigen encounter, B cells of *aly* homozygotes may not receive sufficient activation signals from other cells such as T cells and antigen-presenting cells, resulting in defective responses for both proliferation and germinal center formation.

It has been shown that isotype switching requires a co-stimulatory signal by CD40 ligand on helper T cells and switching isotypes are directed by IL-4, IL-5, IL-10, IFN-γ and TGF-β which are produced by activated T cells (10,11). The CD40–CD40 ligand system is responsible for switching Ig isotypes during T cell-dependent immune responses (29–31). Since *aly/aly* mice produced not only IgM but also IgG1, IgG2b and IgG3 antibodies to the T cell-dependent antigen, the CD40, its ligand system may not be affected in these mutants. Isotype switching to IgG2a was blocked in both T cell-dependent and T cell-independent immune responses in *aly/aly* mice. The analysis of mice deficient in the IFN-γ receptor demonstrated that the efficiency of class switching to the IgG2a isotype was reduced (32). Thus, reduction in IFN-γ production may be responsible for incomplete class switching in the *aly/aly* mutant. Since IFN-γ is produced mainly by antigen-stimulated T helper cells, functions of not only B cells but also T cells may be defective on antigen encounter in the *aly/aly* mutant.

Although *in vivo* lymphocyte functions are defective as described above, it is not clear whether or not the *aly* mutation causes the intrinsic defects in the lymphocyte. It is also demonstrated that the constitutive cytolytic activity of the

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intestinal intraepithelial T lymphocytes is attenuated sharply in *aly/aly* mutants (15). However, this activity is augmented significantly after *in vitro* stimulation with an anti-CD3 mAb. In addition, class switching to IgA took place in *in vitro* culture of *aly* homozygote spleen cells in the presence of conditioning cytokines (our unpublished data). However, repeated immunizations *in vivo* did not induce production of IgA in *aly/aly* mice. Therefore, the *aly* mutation may have something to do with the communication network among lymphocytes and accessory cells.

We speculate that the key defect in the *aly* mutation resides in the signaling cascade upon antigen encounter and the initiation of immune responses. A recent histological study demonstrated that the initial B cell activation by antigens and T cells occurs outside the follicles, i.e. in outer periarteriolar lymphoid sheath and around terminal arterioles (33). On the other hand, it has been well documented that an intact splenic marginal zone is required for an efficient response to T-independent type 2 antigen (34,35). The *aly/aly* spleen has no distinct marginal zone and poorer architecture of periarteriolar lymphoid sheath, suggesting an impaired initial B cell activation on antigen encounter in *aly/aly* mutants, although it remains unclear which cells or which signals are defective in this reaction. Miyawaki *et al.* (12) performed a reciprocal bone marrow transplantation experiment between *aly/aly* and normal mice to examine whether the *aly* gene affects lymphocytes or the lymphoid tissue micro environment. Their data suggest that the *aly* mutation may affect a molecule expressed on both bone marrow-derived cells and bone marrow-independent cells. Such a molecule may be responsible for generation of lymph nodes and Peyer's patches, and have an important role in activation of lymphocytes on antigen encounter. The molecular cloning of the *aly* gene should provide further insights into signaling involved in the immune response.

Abbreviations

DNP	dinitrophenylbenzene
HGG	human γ-globulin
NP	(4-hydroxy-3-nitrophenyl)acetyl
PNA	peanut agglutinin
TGF	transforming growth factor
TNP	trinitrophenylbenzene

Acknowledgements

We thank Dr H. Yoshida (Kyoto University) for his technical advice, Ms H. Ohori for her technical assistance and Ms N. Nakahashi for her help in preparation of the manuscript.

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