# Affinity maturation without germinal centres in lymphotoxin-α-deficient mice

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**Affinity maturation by somatic hypermutation is thought to occur within germinal centres[1–4]. Mice deficient in lymphotoxin-α ($LT\alpha^{-/-}$ mice) have no lymph nodes or Peyer's patches[5,6], and fail to form germinal centres in the spleen[7]. We tested whether germinal centres are essential for maturation of antibody responses to T-cell-dependent antigens. $LT\alpha^{-/-}$ mice immunized with low doses of (4-hydroxy-3-nitrophenyl)acetyl-ovalbumin (NP-OVA) showed dramatically impaired production of high-affinity anti-NP IgG1. However, $LT\alpha^{-/-}$ mice immunized with high doses of NP-OVA, even though they failed to produce germinal centres, manifested a high-affinity anti-NP IgG1 response similar to wild-type mice. Furthermore, when $LT\alpha^{-/-}$ mice were multiply immunized with high doses of NP-OVA, the predominantly expressed anti-NP VH gene segment *VH186.2* showed somatic mutations typical of affinity maturation[8]. Thus, B-cell memory and affinity maturation are not absolutely dependent on the presence of germinal centres.**

The failure of $LT\alpha^{-/-}$ mice to develop germinal centres persists even after multiple immunizations with high doses (200 μg per injection) of the T-cell-dependent antigen NP-OVA adsorbed to alum (Fig. 1*a*, *b*). Flow cytometry using peanut agglutinin (PNA)[4] can quantify germinal-centre B cells after immunization. Wild-type mice showed clear induction of $PNA^{high}$ B cells both after single (data not shown) and multiple immunizations with NP-OVA adsorbed to alum (Fig. 1*c*); in contrast, no $PNA^{high}$ B cells were detected in the $LT\alpha^{-/-}$ mice. Similar results were obtained after two immunizations with another T-cell-dependent antigen keyhole-limpet haemocyanin (KLH), injected without adjuvant (data not shown). Thus, $LT\alpha^{-/-}$ mice fail to form germinal centres even after repeated immunizations with T-cell-dependent antigens, with or without adjuvant.

Both primary and secondary lymphoid follicles characteristically contain follicular dendritic cells (FDCs)[9]. FDCs trap and can retain antigen–antibody complexes for long periods[10], apparently by means of receptors (CR1, CR2 and CR3) for the third complement component[9]. FDCs in spleen follicles can be visualized immunohistochemically by using 8C12, a monoclonal

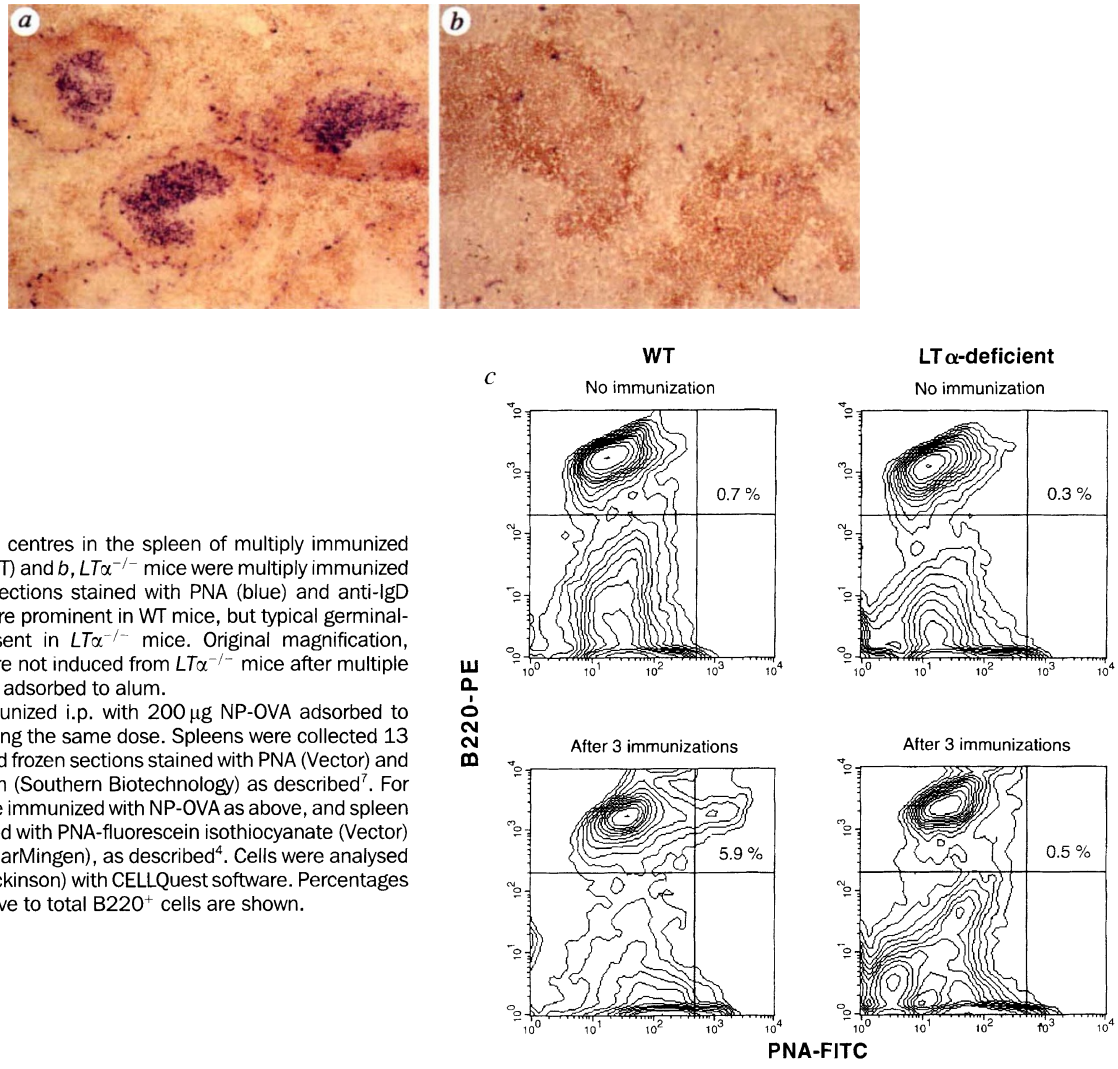


FIG. 1 Absence of germinal centres in the spleen of multiply immunized $LT\alpha^{-/-}$ mice. *a*, Wild-type (WT) and *b*, $LT\alpha^{-/-}$ mice were multiply immunized with NP-OVA, and spleen sections stained with PNA (blue) and anti-IgD (brown). Germinal centres are prominent in WT mice, but typical germinal-centre development is absent in $LT\alpha^{-/-}$ mice. Original magnification, ×100. *c*, $PNA^{high}$ B cells were not induced from $LT\alpha^{-/-}$ mice after multiple immunizations with NP-OVA adsorbed to alum.
METHODS. Mice were immunized i.p. with 200 μg NP-OVA adsorbed to alum, and boosted twice using the same dose. Spleens were collected 13 days after the final boost, and frozen sections stained with PNA (Vector) and polyclonal rat anti-IgD serum (Southern Biotechnology) as described[7]. For flow cytometry (c), mice were immunized with NP-OVA as above, and spleen cell suspensions were stained with PNA-fluorescein isothiocyanate (Vector) and B220-phycoerythrin (PharMingen), as described[4]. Cells were analysed using a FACScan (Becton Dickinson) with CELLQuest software. Percentages of $PNA^{high}$ $B220^{+}$ cells relative to total $B220^{+}$ cells are shown.

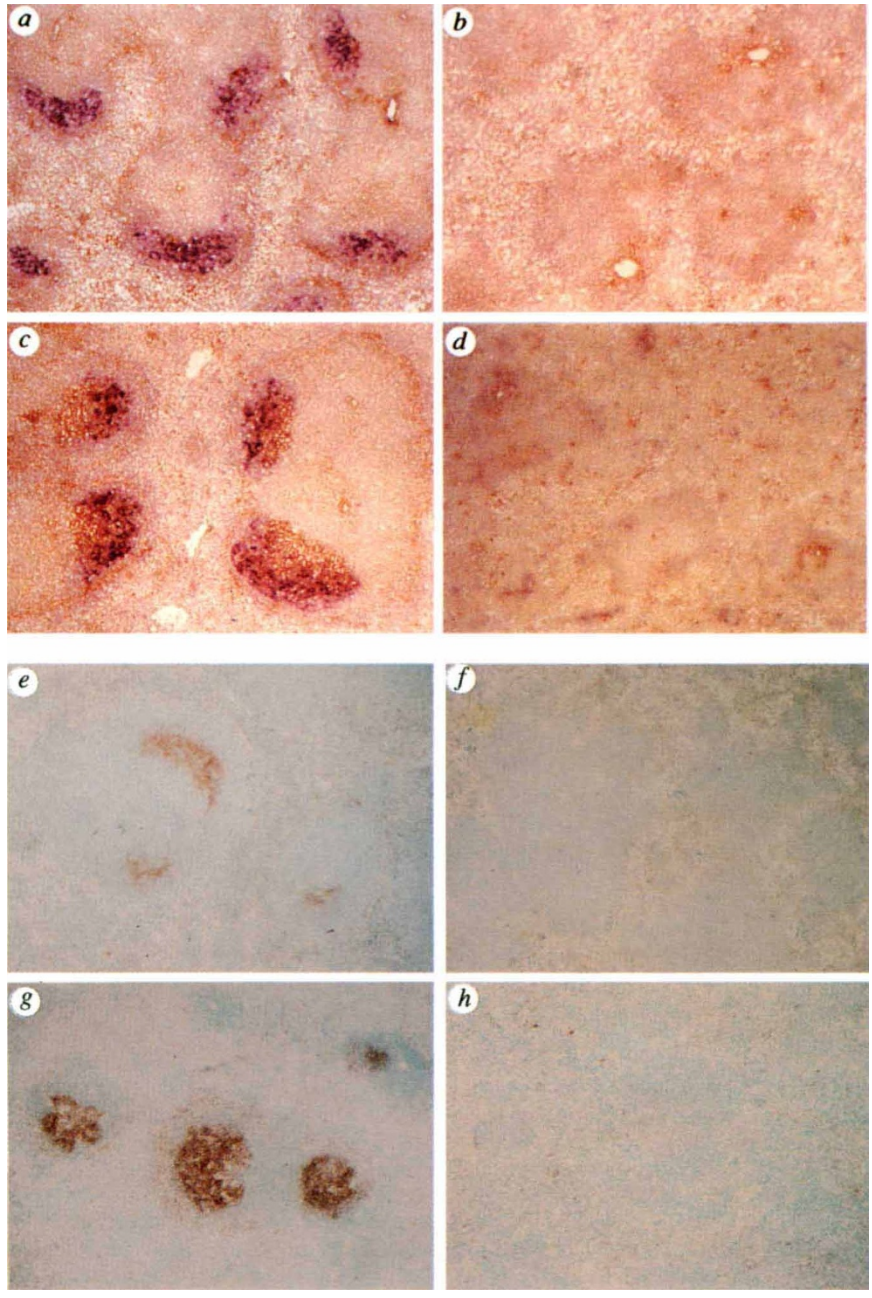


FIG. 2 Organization of FDCs is disturbed in $LT\alpha^{-/-}$ mice. Clustering of FDCs was detected with anti-CR1 monoclonal antibody (mAb) (blue staining) from unimmunized WT mouse spleen (*a*), and occupied the light zone of germinal centres (brown staining with PNA) after immunization with NP-OVA (*c*). $LT\alpha^{-/-}$ spleen showed no staining with anti-CR1 mAb or PNA, before (*b*) or after (*d*) immunization. PAP complexes either injected into the WT mice (*e*) or incubated *in vitro* with sections of WT spleen (*g*) were trapped by the spleen follicles. The spleen follicles of $LT\alpha^{-/-}$ mice failed to show trapping of PAP complexes either injected *in vivo* (*f*) or incubated *in vitro* (*h*). Original magnification, ×100.

METHODS. Immunization of mice with NP-OVA and immunohistochemistry using PNA and anti-CR1 mAb (8C12)[11] were performed as described in Fig. 1. For *e*, *f*, rabbit PAP complex (200 µl) (DAKO A/S; code Z 113, lot 082) was injected intravenously into mice which had been immunized 10 days previously with sheep red blood cells (100 µl of a 10% suspension in PBS, pH 7.4), and spleens were collected 24 h later. Frozen sections of spleen were prepared and further incubated with horseradish peroxidase (HRP) conjugated goat anti-rabbit serum (Southern Biotechnology). After washing in PBS, colour development for bound HRP was performed using diaminobenzidine. For *g*, *h*, immune complex trapping *in vitro* was performed as described[26]. Frozen sections of spleen were incubated with 1:10 diluted mouse PAP (DAKO A/S; code B650, lot 035A) in the presence of 1:5 diluted fresh mouse serum as a source of complement. After washing in PBS, colour was developed as described above.

antibody specific for murine CR1 (ref. 11). In spleen of naive wild-type mice, FDC reticula were prominently stained (Fig. 2*a*); after immunization, the FDC constituted the germinal-centre light zone (Fig. 2*c*). In contrast, naive (Fig. 2*b*) and immunized (Fig. 2*d*) $LT\alpha^{-/-}$ mice showed no clusters of 8C12-staining cells. Failure of normal FDC organization was further demonstrated by analysis of immune complex trapping. Spleen follicles in wild-type mice trapped preformed horseradish peroxidase–anti-peroxidase (PAP) complexes either *in vivo* or *in vitro*, at sites corresponding to the FDC reticula (Fig. 2*e*, *g*). Immune complex trapping was not detected in $LT\alpha^{-/-}$ mice (Fig. 2*f*, *h*). Thus in $LT\alpha^{-/-}$ mice, organized FDC structure, a major morphological characteristic of germinal centres, is absent.

We tested whether germinal centres are essential for the maturation of antibody responses to T-cell-dependent antigens. $LT\alpha^{-/-}$ and wild-type mice that had not been immunized showed similar levels of total serum IgM and IgG (data not shown). Anti-NP antibodies were measured using an enzyme-linked immunosorbent assay (ELISA) following intraperitoneal immunization of mice with NP-OVA. When the ELISA plate was coated with densely NP-haptenated bovine serum albumin ($NP_{13}$-BSA), both high- and low-affinity anti-NP antibodies were bound. When the plate was coated with sparsely haptenated BSA ($NP_{2.5}$-BSA), only high-affinity anti-NP antibodies were bound. Therefore, the appearance of $NP_{2.5}$-binding antibodies and an increased ratio of $NP_{2.5}$-binding/$NP_{13}$-binding antibody demonstrate affinity maturation[12,13]. When immunized twice with either 1 or 5 µg of NP-OVA, all wild-type mice produced NP-specific IgG1 antibody and showed affinity maturation (Fig. 3*a*); by day 28, the average ratio of $NP_{2.5}$/$NP_{13}$-binding antibodies was 0.8. In contrast, only two of six $LT\alpha^{-/-}$ mice produced detectable anti-NP IgG1 by day 28, with no evidence of high-affinity antibody. The $LT\alpha^{-/-}$ mice showed anti-NP IgM responses equal to or greater than those of wild-type mice (data not shown). Thus, the impaired IgG1 response in $LT\alpha^{-/-}$ mice immunized with low doses of antigen did not result from a defect in antigen delivery.

High doses of NP-OVA (200 µg) overcame this defective humoral response. After high-dose immunization, $LT\alpha^{-/-}$ mice

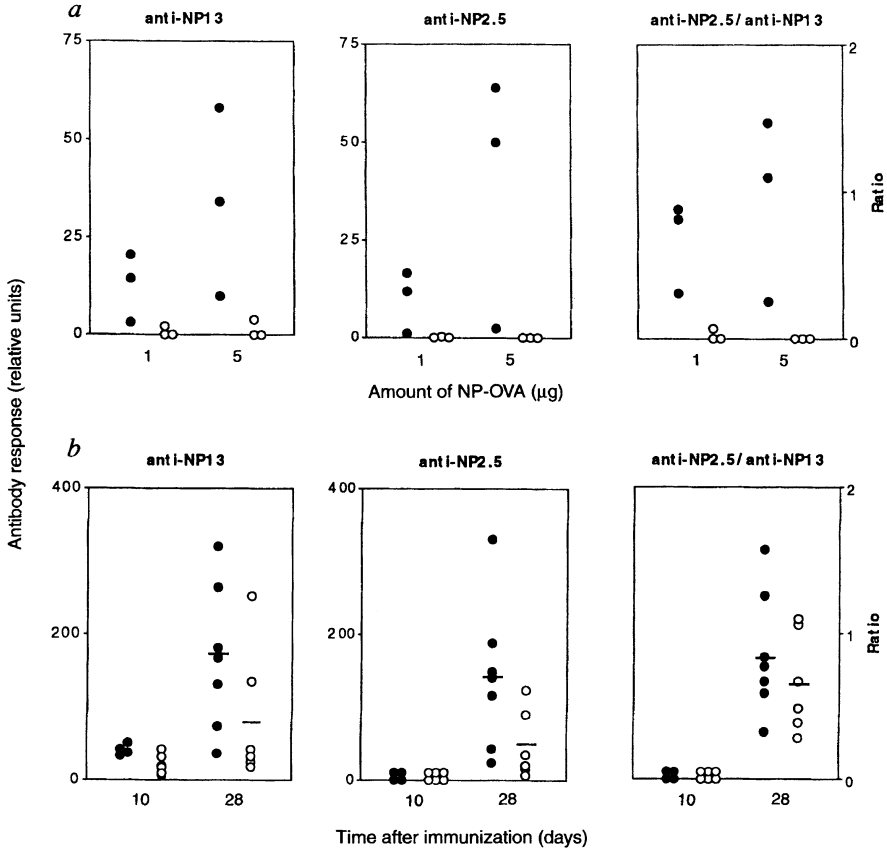


FIG. 3 Antigen-specific antibody responses in $LT\alpha^{-/-}$ mice vary according to the dosage of immunogen. *a*, Antibody response (amount of NP-specific serum IgG1) at day 28 in $LT\alpha^{-/-}$ mice following immunization with a low dose of NP-OVA. *b*, Antibody response in $LT\alpha^{-/-}$ mice after immunization with a high dose of NP-OVA. WT mice, filled circles; $LT\alpha^{-/-}$ mice, open circles. METHODS. Both WT and $LT\alpha^{-/-}$ mice were immunized i.p. with either: *a*, low doses (1 or 5 μg per injection), or *b*, high doses (200 μg per injection) of NP-OVA adsorbed to alum. Mice were boosted 21 days later using the same doses of antigen. Sera were collected 10 and 28 days after the initial immunization. NP-specific IgG1 antibody of low and high affinity was detected by ELISA with $NP_{13}$-BSA and $NP_{2.5}$-BSA coated plates, respectively[12,13]. Antibodies bound to the plates were detected using a goat anti-mouse IgG1 antibody conjugated with HRP (Southern Biotechnology). The quantities of NP-specific serum IgG1 in each experiment were expressed in relative units compared to a standard hyperimmune mouse serum[29]; we defined 100 units as the quantity of anti-NP antibody in a standard hyperimmune serum. When both anti-$NP_{2.5}$ and anti-$NP_{13}$ antibodies were undetectable, the ratio of anti-$NP_{2.5}$/anti-$NP_{13}$ was defined as 0.

produced similar amounts of NP-specific IgG1 compared to wild-type mice, demonstrating that isotype switching can occur in the absence of germinal centres (Fig. 3*b*). Furthermore, the ratio of IgG1 antibody bound to $NP_{2.5}$-BSA and $NP_{13}$-BSA suggested similar acquisition of high-affinity antibody in wild-type and $LT\alpha^{-/-}$ mice. Thus affinity maturation is not absolutely dependent on germinal centres.

The locations of anti-NP IgG1-producing cells in high-dose NP-OVA-immunized wild-type and $LT\alpha^{-/-}$ mice were investigated using the ELIspot assay[13]. Seven days after a single immunization, $LT\alpha^{-/-}$ mice showed no detectable anti-NP IgG1-producing cells in either peripheral blood or resident peritoneal cells (<1 anti-NP-producing cell per $10^6$ leukocytes). Anti-NP IgG1-producing cells were detected at 10 per $10^5$ cells in spleen. Wild-type mice showed no anti-NP IgG1-producing cells in peripheral blood or peritoneal cells, and 8 anti-NP IgG1-producing cells per $10^5$ cells in spleen. These data suggest that the maturation of the anti-NP B-cell response occurs primarily in the spleen in both wild-type and $LT\alpha^{-/-}$ mice.

During the primary response to NP, the combination of λ1 light chain and the heavy-chain variable region gene *VH186.2* predominates[2,8,14–16]. This VH gene also predominates in secondary responses, but in somatically mutated forms[8,15,17–19]. Mutation of Trp to Leu at codon 33 in the first complementarity determining region (CDR1) of the *VH186.2* gene is highly correlated with acquisition of higher affinity for NP[8,20]. We examined the sequences of *VH186.2* IgG1 transcripts expressed in spleen after immunization with NP-OVA and found that immunized and boosted wild-type mice showed somatic mutations scattered throughout the *VH186.2* segment (Fig. 4*a*). Codon 33 in the CDR1 region showed mutation from Trp to Leu in 14 of 18 independent sequences, indicating affinity maturation by somatic mutation. Similarly immunized and boosted $LT\alpha^{-/-}$ mice also showed somatic mutation of the variable regions in RNA transcribed from this gene (Fig. 4*b*). The total number of somatically mutated nucleotides was only 30% of that in wild-type mice (Fig. 4*c*). Nevertheless, 9 of 18 independent sequences showed the codon 33 Trp to Leu mutation (Fig. 4*b*). Thus, although somatic mutation was reduced in the $LT\alpha^{-/-}$ mice, selection of cells expressing high-affinity variants occurred effectively. In addition, the mutations observed showed a similar pattern of strand bias, preference for transitions rather than transversions, and favouring of mutations at AGC/T sequences, as previously observed[21,22], suggesting that the mutational mechanism in the $LT\alpha^{-/-}$ mice has the same properties as that in wild-type mice.

The pattern of replacement and silent mutations in the expressed *VH186.2* sequences provides additional evidence of selection following mutation (Fig. 4*c*). If somatic mutations accumulated randomly, then the expected ratio of replacement to silent mutations in the CDR and the framework regions would be 4.9 and 2.6, respectively[13,18]. In both $LT\alpha^{-/-}$ and wild-type mice, the observed replacement/silent value in CDR1 and CDR2 is higher than that expected from random mutagenesis, whereas the value observed in the framework regions approximates the expected value. These results suggest that, as in wild-type mice, somatic mutation in $LT\alpha^{-/-}$ mice is an antigen-driven process.

The absence of germinal centres in $LT\alpha^{-/-}$ mice is associated with failure to organize clusters of FDCs. Differentiation and/or organization of FDCs is thought to depend on interactions with both B and T cells[23–26]. Thus, disturbed organization of FDCs in $LT\alpha^{-/-}$ mice might not be the primary cellular defect that determines absence of germinal centres. However, the type I tumour-necrosis-factor receptor (TNFR-I), which binds the soluble LTα homotrimer, is expressed highly on FDCs in the spleen[27], and mice deficient for TNFR-I also fail to form both germinal centres[7] and organized FDC clusters (data not shown). LTα-deficiency may result in failure to deliver a TNFR-I-mediated signal that is required for recruitment of FDCs, which in turn, may lead to failure to form germinal centres.

It is striking that high doses of antigen can activate the affinity maturation process even in the absence of germinal centres. Both somatic mutations of immunoglobulin genes and selection of

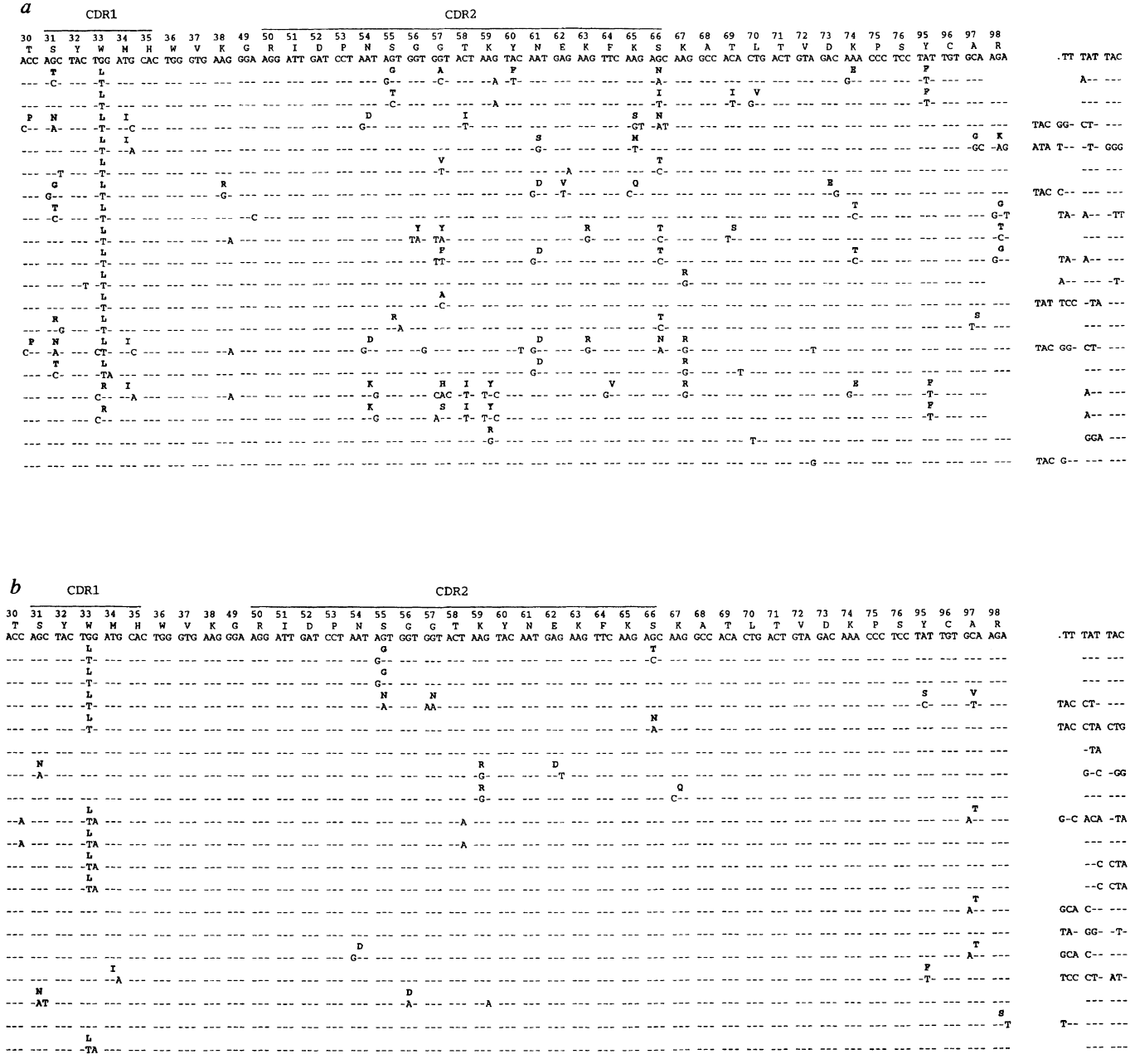

FIG. 4 Somatic mutation of the *VH186.2* gene in NP-immunized mice. Selected codons of the CDR1 and CDR2 domains of the germline *VH186.2* gene joined to DFL16.1 are shown on the top lines with the amino-acid translation using the single-letter code above. Expressed sequences from twice-boosted WT (*a*) and *LTα*$^{-/-}$ mice (*b*) are shown below. Dashes indicate nucleotides identical to the germline sequence. *c*, Patterns of somatic mutations were determined by pooling data from 18 clones obtained from two WT and two *LTα*$^{-/-}$ mice. Asterisk indicates the ratio of replacement (R) to silent (S) mutations; expected R/S values are if mutagenesis was random.

METHODS. Mice were immunized three times with 200 μg NP-OVA adsorbed to alum. RNA was extracted from spleens 7 days after the third immunization using RNAzol B (Biotecx Laboratories). cDNA was synthesized from 0.2 μg of total RNA using oligo(dT) primed reverse transcription, and subjected to nested polymerase chain reaction (PCR) to recover *VH186.2* gene sequences joined to the IgG1 constant region. In the first reaction, the 5′ primer was CATGCTCTTCTTGGCAGCAACAGC (for *VH186.2*), and the 3′ primer was GTGCACACCGCTGGACAGGGATCC (for Cγ1). PCR was performed for 30 cycles of 1 min at 94 °C, 2 min at 55 °C, and 3 min at 72 °C. The second reaction used 5′ primer CAGGTCCAACTGCAGCAG and 3′ primer AGTTTGGGCAGCAGA for 30 cycles under the same conditions. Amplified fragments were cloned in pCRII (Invitrogen), and randomly picked transformants were sequenced using Sequenase (USB). Control sequences from before the induction of somatic mutation demonstrated that base changes due to PCR error were <0.2 nucleotides per clone. Without immunization, no *VH186.2* transcripts were amplified from either WT or *LTα*$^{-/-}$ mice (data not shown).

*c*

| | Mutation frequency | R/S* | |
|---|---|---|---|
| | | CDR (expected: 4.9) | Framework region (expected: 2.6) |
| WT | 9.0 per clone | 9.9 | 3.1 |
| *LTα*$^{-/-}$ | 3.2 per clone | 7.7 | 2.3 |

clones producing high-affinity antibodies were detected in $LT\alpha^{-/-}$ mice. Thus, this germinal-centre-independent mechanism possesses the essential characteristics of the germinal-centre-dependent affinity maturation process. It has been suggested that the selection of B lymphocytes expressing high-affinity mutants of their immunoglobulin chains occurs normally within the germinal centre by competition for antigen displayed on the surfaces of FDCs, and that B cells that are not selected die by apoptosis[1,9,28]. Selection of high-affinity antibody-producing cells in $LT\alpha^{-/-}$ mice in the absence of either germinal centres or organized FDC clusters appears inconsistent with this model. However, although $LT\alpha^{-/-}$ mice contain no recognizable FDC clusters, there may be FDCs dispersed throughout the splenic follicles in sufficient numbers to support the selection process. Alternatively, there may be other cell populations that can substitute for FDCs. □

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# 'Anaphase' and cytokinesis in the absence of chromosomes

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**ANAPHASE and cytokinesis are key processes in the segregation of replicated chromosomes to the daughter cells: in anaphase, chromosomes move apart; in cytokinesis, a cleavage furrow forms midway between the separated chromosomes. Some evidence suggests that chromosomes may be involved both in controlling the timing of anaphase onset[1–3] and in dictating the position of the cleavage furrow[3]. Other evidence indicates that the controlling mechanisms are intrinsic to the spindle and the cell[4–7]. Here we test these possibilities in grasshopper spermatocytes by observing spindles and cells after removal of chromosomes. We found that both anaphase and cytokinesis occur independently of chromosomes: stage-specific changes occur at an appropriate time and in the correct way, despite the absence of chromosomes. This finding is particularly noteworthy because chromosomes have an important impact on spindle microtubule assembly[8,9] and the timing of anaphase onset[10] in these cells.**

Spermatocytes of the grasshoppers *Chortophaga australior* and *Melanoplus sanguinipes* have eleven bivalents and an X chromosome. Although chromosomes are required for spindle assembly in these cells[9], once a spindle forms, the absence of chromosomes does not affect the integrity of the spindle[8]. We selected cells with a well-formed spindle and removed all the chromosomes by micromanipulation. We then followed spindle behaviour using video-enhanced polarization microscopy.

In cells from which all chromosomes were removed, the spindle changed with time much like a normal spindle with a normal set of chromosomes (Fig. 1*a*, *b*). At a stage approximating metaphase, a bipolar spindle was perfectly maintained (Fig. 1*b*, −112 and −14 min). Spindle microtubules, however, frequently grouped into bundles that either dispersed over time or elongated toward the cell equator and then shortened back to the poles (Fig. 2). At the time when normal cells entered anaphase, the cell devoid of chromosomes also progressed into 'anaphase' (Fig. 1*a*, *b*; 0 min). For cells lacking chromosomes, the onset of 'anaphase' was marked by the appearance of a gap in the spindle (Fig. 1*b*, bracket, 0 min), that is, by a much-reduced microtubule density at the equator. As anaphase progressed, this gap expanded toward the spindle poles (0 min onwards). Nine of ten cells without chromosomes entered 'anaphase' within 15 min of the time when anaphase commenced in control cells which had not been operated on. For ease of comparison of events in experimental and control cells, the onset of anaphase was set to 0 min (Figs 1 and 3). The rate of half-spindle shortening, determined by measurement of gap expansion in *Chortophaga australior*, averaged $0.47\,\mu m\,min^{-1} \pm 0.11$ (95% confidence interval; $n = 5$), a rate similar to that for chromosome–to–pole movement (anaphase A) in a normal spindle ($0.58\,\mu m\,min^{-1} \pm 0.05$; $n = 5$). Elongation of the spindle (anaphase B), so characteristic of normal anaphase, occurred on time in cells lacking chromosomes (Fig. 1*a*, *b*; 3–14 min) at a rate ($0.41\,\mu m\,min^{-1} \pm 0.14$; $n = 4$) similar to that of the cells with chromosomes ($0.53\,\mu m\,min^{-1} \pm 0.25$; $n = 4$; rate of spindle elongation is variable both in controls and cells without chromosomes). Finally, normal anaphase is marked by a redistribution of mitochondria (Fig. 1*a*, m), and an analogous process occurred on schedule in spindles without chromosomes (Fig. 1*b*).

Cytokinesis in the absence of chromosomes also appeared remarkably normal. At the time when control cells entered cytokinesis (Fig. 3*a*), a well defined cleavage furrow appeared in cells without chromosomes (Fig. 3*b*, arrow). The furrow was initiated precisely midway between the two spindle poles. Contraction of the furrow divided the cell into two cells without nuclei (Fig. 3*b*).

Our results agree with the early discoveries that echinoderm embryos can traverse the cell cycle repeatedly in the absence of nuclei[4–6,11]. However, even though centrosomes duplicate during each cell cycle in these embryonic cells, a bipolar spindle does not appear[5]. In our experiments, we did not remove the chromosomes from the cell until a bipolar spindle had formed. Stage-specific changes in the spindles and the cells are revealed for the first time in the absence of chromosomes. We show that 'anaphase' and cytokinesis can occur normally and punctually in cells containing no chromosomes. This implies that some controlling mechanisms for these events are intrinsic to the spindle and the cell, but not the chromosomes. In the absence of chromosomes, the cell-cycle clock ticks on, staying precisely on time.

It has been suggested that ubiquitin-mediated destruction of a 'glue protein' that holds replicated chromosomes together may be needed to initiate anaphase[1,2]. Although such a process probably