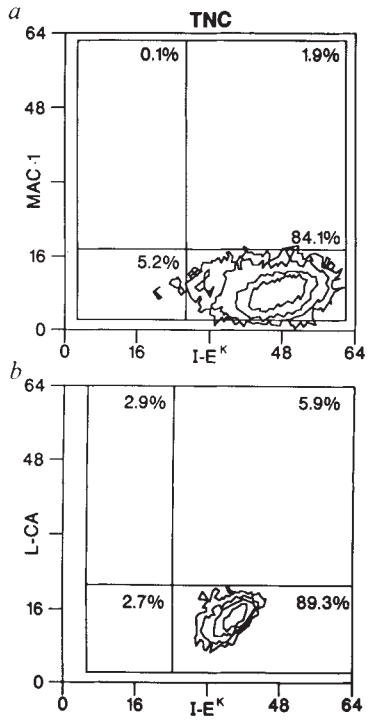

Fig. 4 Two-colour flow cytometric analysis of TNC surface marker expression. Each FACS profile is from TNC isolated from a total of 60 CBA/J mice: in *a*, 23,891 cells and in *b*, 8,619 cells were analysed using a Becton-Dickinson FACS 440. The TNC population was isolated as described in Fig. 2. This purified cell population was treated sequentially with anti-Thy 1.2 (AT83A, from F. Fitch), then guinea pig complement (Colorado Serum Co.) to remove any free thymocytes. The TNC were labelled with either rat-anti-MAC-1 (M1/70, from the American Type Culture Collection[25] or rat-anti-leukocyte common antigen (L-CA) (I3/2; ref. 26), plus biotinylated mouse-anti-I-$E^{k/d}$ (14-4-4S; ref. 27). Secondary reagents used were fluorescein isothiocyanate (FITC)-conjugated goat anti-rat immunoglobulin cross-absorbed against mouse immunoglobulin (Cooper Biomedical) and streptavidin-phycoerythrin (PE)-conjugate (Chromoprobe). In these contour plots, PE and FITC fluorescence are plotted on the *x* and *y* axis respectively. Lines used to define positive versus negative cells were set against the outermost contour of controls where the primary antibodies were omitted. Both the T-ROS and Mϕ/DC population were positive for MAC-1 and L-CA (data not shown).

ized its cell-surface phenotype by two-colour fluorescence activated cell sorter (FACS) analysis. As shown in Fig. 4, the TNC population is class II-positive, but essentially negative for both leukocyte common antigen (a marker for bone marrow-derived cells) and MAC-1 (a marker for both thymic Mϕ and DC, but not for TNC)[14,15]. These results indicate that the antigen presentation seen in our TNC preparation is not due to contamination from a bone marrow-derived APC. This raises the question of how the Hb reaches the epithelial cells. One possibility suggested by data reviewed in ref. 16 indicates that antigens can bypass the blood-thymus barrier and enter the cortical region by a transcapsular route.

Our experiments demonstrate that non-MHC self proteins are processed and presented by fetal thymic APCs at the time when an individual is acquiring tolerance to self. These findings have been extended to show that the APC responsible for this presentation of self molecules is not limited to one cell type, but instead can be accomplished by at least three distinct thymic cell populations. Two of these cell populations are traditional APCs, the Mϕ and the DC, but the third type, the cortical epithelial cell, has not been shown before to be able to present antigen. This novel finding forces us to revise our theories on the role of cortical epithelial cells in self tolerance and T-cell development[17,18]. It must now be considered that for the self antigen Hb, negative as well as positive selection may be occurring in the cortex, directed by non-bone marrow-derived cells. It will be interesting to see if tolerance to other self-proteins also follows this pattern.

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Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes

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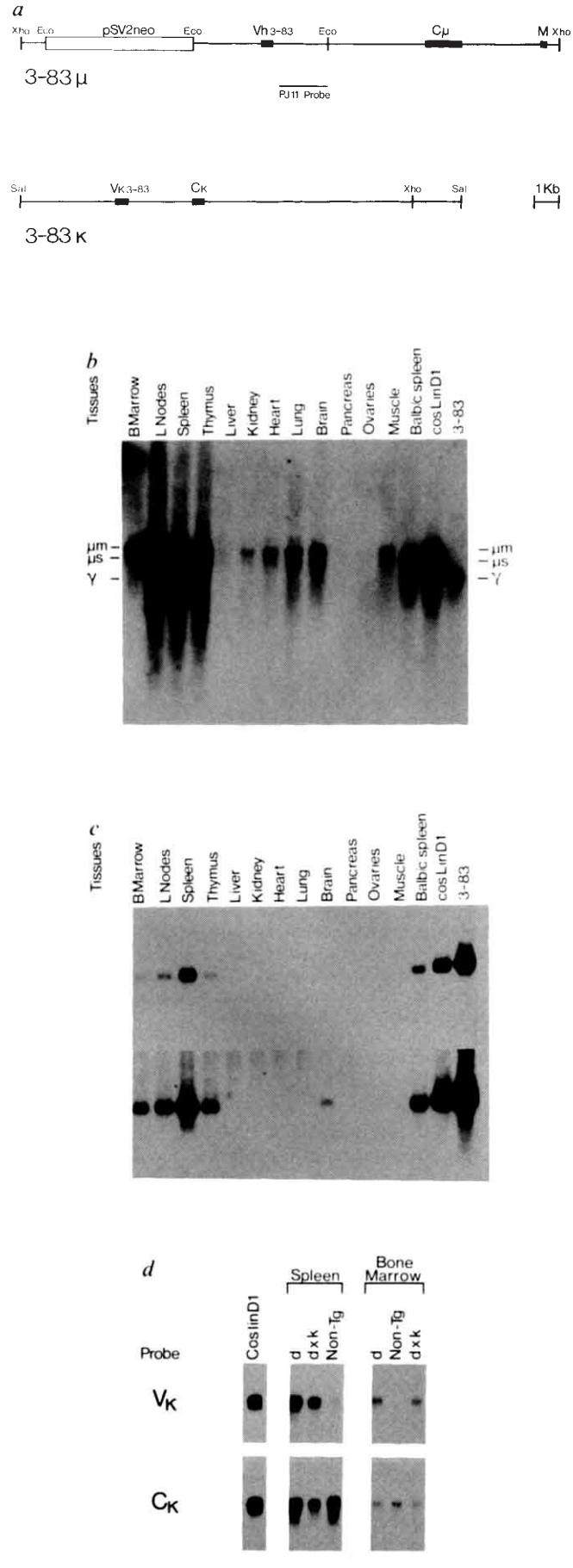
B lymphocytes can be rendered specifically unresponsive to antigen by experimental manipulation *in vivo* and *in vitro*[1–6], but it remains unclear whether or not natural tolerance involves B-cell tolerance because B cells are controlled by T lymphocytes, and in their absence respond poorly to antigen (reviewed in ref. 7). In addition, autoantibody-producing cells can be found in normal mice and their formation is enhanced by B-cell mitogens such as lipopolysaccharides[8–12]. We have studied B-cell tolerance in transgenic mice using genes for IgM anti-H-2^k MHC class I antibody. In H-2^d transgenic mice about 25–50% of the splenic B cells bear membrane immunoglobulin of this specificity, and abundant serum IgM encoded by the transgenes is produced. In contrast, H-2^k × H-2^d (H-2-d/k) transgenic mice lack B cells bearing the anti-H-2^k idiotype and contain no detectable serum anti-H-2^k antibody, suggesting that very large numbers of autospecific B cells can be controlled by clonal deletion.

We cloned genes for the transgenic (Tg) anti-H-$2K^kD^k$ antibody from the DNA of the BALB/c IgG-producing hybridoma 3-83 (ref. 13), and constructed a chimaeric heavy-chain gene using a mouse Cμ constant-region DNA clone (see Fig. 1*a*). The founder male Tg mouse was derived from an embryo that was homozygous for the H-2^d haplotype (H-2-d/d). Its serum had a high anti-H-2^k cytotoxic antibody titre and contained at least 50 μg ml^{-1} of the 3-83 idiotype (id), as recognized by the rat monoclonal anti-idiotypic antibodies 35B and 54.1 that bind



◀ **Fig. 1** Production and RNA analysis of transgenic mice. *a*, Genes used for generation of transgenic mice. Open box indicates the pSV2neo vector sequences[21], and the pJ11 (ref. 22) probe is indicated. *b*, *c* And *d*, Northern analysis of H-2^d Tg tissues using 3-83 V_H, V_κ and C_κ probes: *b*, V_H probe. 10 μg RNA (3-83 RNA, 1 μg) was loaded in each lane. Cos Lin D1 was a myeloma transfectant expressing chimaeric 3-83 genes; *c*, V_κ probe. As in *b*, except that only 5 μg lymph node RNA was run. The two panels show 1- and 7-day exposures of the same blot; *d*, 5 μg RNA per lane. The filter was first hybridized with V_κ probe (top), exposed, stripped and rehybridized with C_κ probe.

Methods. The VDJ_H segment was isolated from λgt-wes *Eco*RI library using J-Cμ intron-specific probe pJ11. The intact VC_κ 3-83 gene was isolated from an EMBL3 *Sau*3A partial digest library. The VDJ_H 3-83 was ligated into plasmid pRμR1 containing the genomic Cμ gene. To prepare DNA for microinjection, 3-83μ plasmid was linearized at the unique *Xho*1site. The 3-83κ DNA was excised with *Sal*I, which cuts in polylinker regions flanking the insert, and the insert was isolated by electrophoresis through low-melting-temperature agarose. Transgenic mice were produced as described[23,24] from DBA/2 × B10D2F_2 zygotes: about 2 pl DNA solution containing about 500 molecules[25] were microinjected. Purified RNAs were electrophoresed through agarose-formaldehyde gels and transferred to nitrocellulose[26]. Hybridization was at 42 °C in 50% formamide, 1 M NaCl, 10 × Denhardt's solution, 50 mM Tris-HCl, *p*H 7.5, 0.1% $Na_4P_2O_7$, 1% SDS, 150 μg ml^{-1} salmon sperm DNA. The V_H probe was a 400-base pair *Eco*RV-*Bst*EII fragment that included most of the coding sequence. The V_κ probe was a 250-base pair *Hin*dIII-*Bam*HI fragment encompassing the 5′ portion of the gene. The C_κ probe was a *Hpa*I-*Bgl*II fragment that included most of the coding sequence. The final wash was for 30 min in 0.3 × SSC, 0.1% SDS at 65 °C.

an epitope formed by the combination of the 3-83 heavy and light chains[14].

Mice of this transgenic line (designated Tol 1) have 8–10 copies of the microinjected genes, which are apparently integrated at a single locus in an autosome. The 3-83μ messenger RNA was found at high concentrations in lymphoid organs and present in low amounts in several other tissues (Fig. 1*b*), whereas the 3-83 kappa mRNA expression was limited to lymphoid tissues, except in the brain, where it was present at a low level (Fig. 1*c*). The transgenic μ message is expressed in non-lymphoid cells and at in very high amounts in the thymus. It is likely that T cells express large amounts of this message. Two-colour immunofluorescence analysis showed that in H-2-d/d Tg spleen the 3-83 idiotype is found only on B cells (Fig. 2*a*), indicating a tissue-specific expression of the 3-83 IgM protein.

To determine whether the presence of the H-2^k class I antigens affects 3-83 protein expression, we analysed three litters of [(BALB/c (H-2^d) × BALB/k (H-2^k)] × Tol 1 (H-2^d) mice (Table 1). The segregation data showing the expected mendelian ratios, and the large litter sizes (8, 9 and 9 offspring per (BALB/c × BALB/k) F_1 mother respectively), all suggest that no death occurred among H-2-d/k embryos bearing the transgenes. H-2-d/d Tg sera contained high serum concentrations of 3-83 id, which was not detectable in H-2-d/k Tg or in any of the non-Tg littermates, levels being at least 200-fold less (Table 1). 3-83 id made up 61.6% of the total IgM in H-2^d Tg mice. In H-2-d/k Tg mice the IgM levels in the sera were reduced to only 26% of that of the H-2^d Tg mice.

To determine the basis for the apparent tolerance in the H-2-d/k Tg mice, we analysed non-Tg, H-2-d/d Tg and H-2-d/k Tg spleen and bone-marrow cells by cell-surface immunofluorescence (Fig. 2). A quarter to a half of the splenic B cells in H-2-d/d Tg bear the 3-83 id, whereas id-positive cells were undetectable in the H-2-d/k Tg mice; the B cells with the predetermined transgene-encoded anti-self specificity were absent. Non-Tg mice were completely id-negative. We found 3-83 id-positive cells in the spleen, lymph nodes and bone marrow, but not in the thymus of H-2-d/d Tg mice, whereas H-2-d/k Tg mice lacked idiotype-bearing cells in all these tissues. Given the background levels of staining we can be confident that the

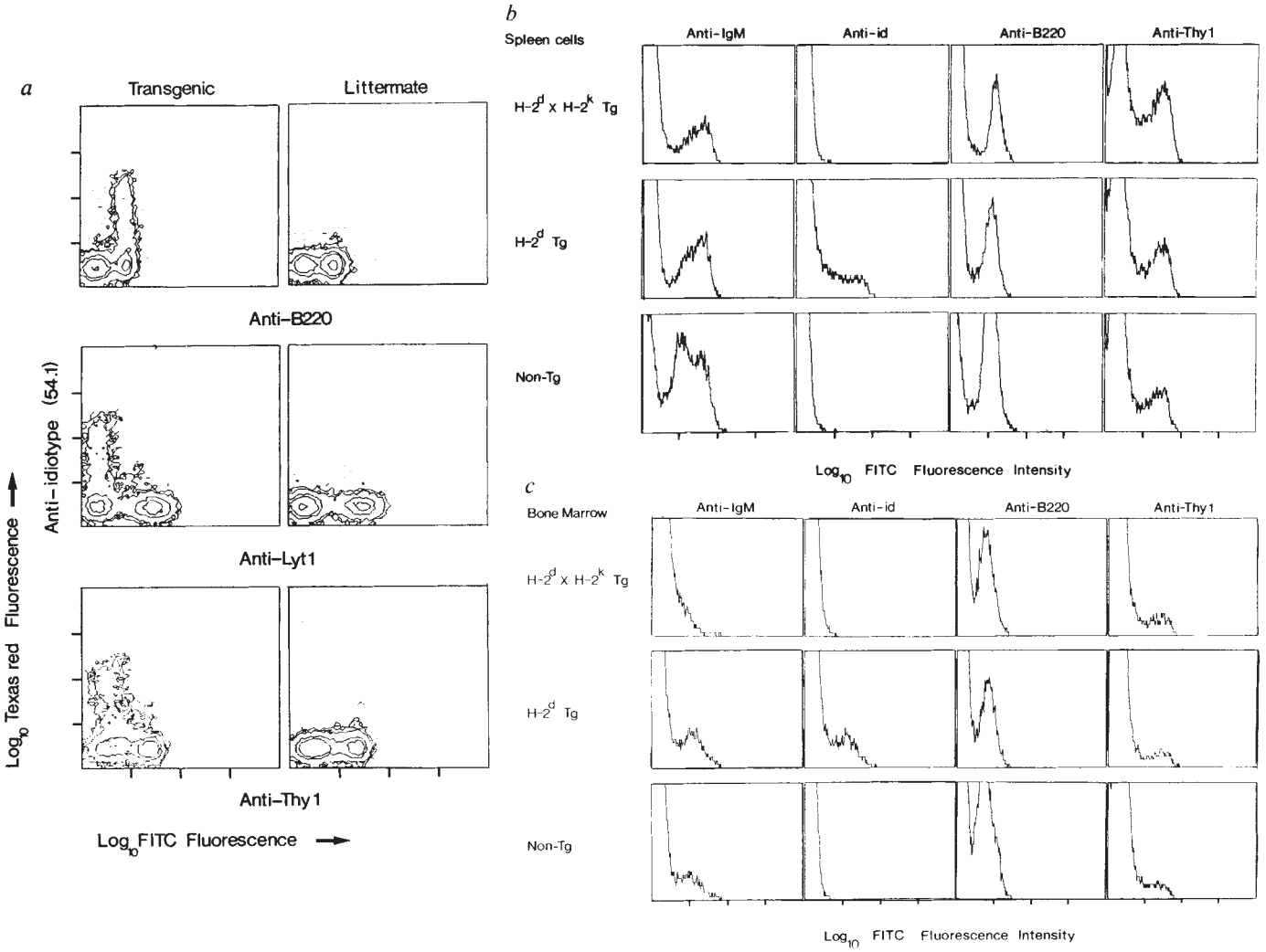


Fig. 2 Flow cytometry analysis. *a*, Two-colour staining of H-2-d/d Tg and control littermate spleen cells. Cells were first incubated with a mixture of biotin-54.1 and fluoroscein isothiocyanate (FITC)-coupled antibodies washed and incubated with Texas red-avidin. Samples were analysed using a Becton-Dickinson fluorescence-activated cell-sorting FACS 440 machine. *b* and *c*, Cells were stained with monoclonal rat anti-IgM (M41) or anti-idiotype (54.1) followed by FITC-labelled mouse anti-rat IgG, or with directly coupled FITC-anti-B220 (RA3-3A1) or FITC-anti-Thyl. Negative control staining with BAG1 (a rat IgG1 monoclonal) gave a result virtually identical to that achieved with anti-idiotype staining of non-transgenic cells. Samples were analysed on a Becton-Dickinson FACSCAN machine.
Methods. All samples were depleted of erythrocytes by treatment in Gey's solution. Dilutant and wash solution in all cases was buffer A (see legend to Table 1). Analysis in *b* and *c* was in buffer A supplemented with 5 μg ml^{-1} propidium iodide to help exclude dead cells from analysis.

frequency of 3-83 id-bearing cells in the H-2-d/k Tg spleens was at least 50-fold lower than in H-2-d/d spleens.

It is unlikely that the capping of cell-surface idiotype bound to H-2^k in H-2-d/k Tg mice would explain the lack of id-positive cells in the spleens of these mice, because in all cases the percentage of IgM-bearing cells closely matched the percentage of cells staining with an independent B-cell marker (B220) and the density of IgM on H-2-d/k and H-2-d/d Tg B cells was comparable (Fig. 2*b*). By contrast, the staining pattern seen with H-2-d/k Tg bone marrow cells (Fig. 2*c*) with its reduced density of IgM and low % IgM:B220 ratio indicates that modulation of cell surface IgM could have occurred, probably after binding to H-2^k class I molecules. We note in this regard that surface immunoglobulin is rapidly capped and then internalized, or is shed within minutes after cross-linking, and that bone-marrow B cells in particular fail to re-express surface immunoglobulin. This has been proposed as a model system for tolerance[15].

To confirm that the lack of 3-83 id in the sera of H-2-d/k Tg mice was due to tolerance rather than to adsorption to H-2^k class I molecules, we analysed frozen sections of spleens for cytoplasmic expression of 3-83 id and IgM using two-colour immunofluorescence. A large fraction of the IgM-producing plasma cells in the H-2-d/d mice produce 3-83 id, whereas almost none of those in H-2-d/k mice were idiotype-positive (Fig. 3). These experiments also confirmed the lack of surface idiotype-positive cells in H-2-d/k Tg and the presence of such cells as a subset of IgM-bearing cells in the H-2-d/d Tg mice. Taken together, these results indicate that 3-83 id-producing cells in the H-2-d/k mice were largely eliminated or excluded from the spleen. The deletion probably occurred at some time shortly after they first expressed their immunoglobulin receptors, so most of them never developed into antibody-secreting cells. This would support reports demonstrating functional inactivation of B cells in tolerant animals[2,3,5].

Despite the absence of 3-83 id-protein expression, H-2^k Tg spleen continues to produce Tg light chain (Fig. 1*d*) and heavy chain mRNA, although levels are reduced compared with H-2^d Tg. As idiotype absorption is ruled out, it seems that the RNA is produced either by non-B cells or as a result of B cells expressing the transgenes which have either mutated these genes or are producing functional immunoglobulin protein chains in addition; these would be encoded by endogenous genes, giving rise to idiotype-negative molecules when paired with Tg chains. Another possibility is that a small, but highly metabolically

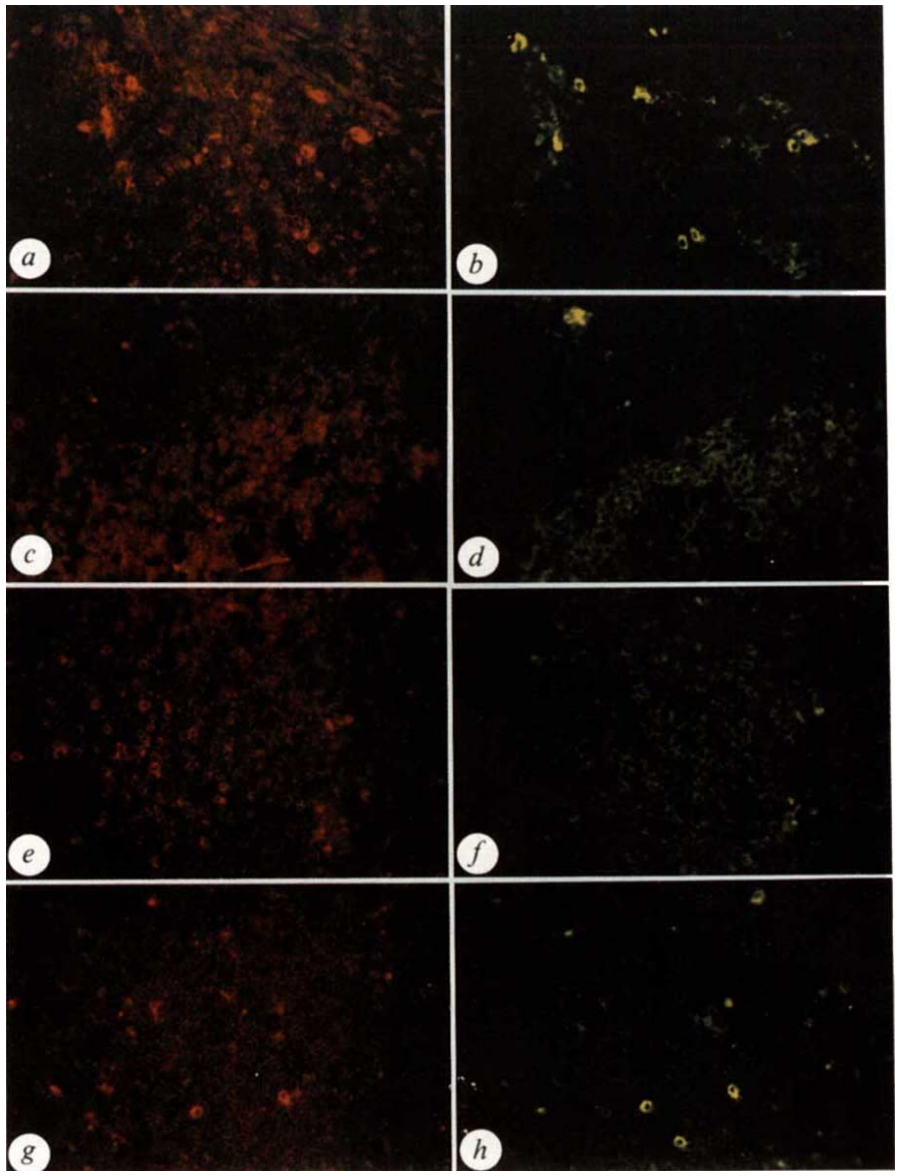


Fig. 3 Two-colour immunofluorescence. *a*, H-2-d/d spleen stained with anti-idiotype; *b*, same section as in *a*, stained with anti-μ; *c*, H-2-d/k spleen anti-idiotype; *d*, same section as in *c*, anti-μ; *e*, field containing a B-cell area from H-2-d/d spleen, anti-idiotype; *f*, same section as in *e*, anti-μ; *g*, H-2-d/d thymus, anti-id; *h*, same as *g*, anti-μ.
Methods. Frozen sections from H-2-d/d spleen (*a*, *b*, *e* and *f*); H-2-d/k spleen (*c*, *d*); H-2-d/d thymus (*g*, *h*) were incubated for 30 min at 4° with a mixture of FITC-rat-anti-IgM and biotin-54.1 in PBS, washed in PBS and incubated with Texas-red avidin for 30 min at 4°. Photographs were taken through a Leitz fluorescence microscope at magnification 360× using the appropriate filters to detect Texas red (*a*, *c*, *e*, *g*) or FITC (*b*, *d*, *f*, *h*) fluorescence.

active, population of transgene-expressing B cells persists.

In all H-2-d/d Tg animals tested so far, a large fraction of the splenic B cells (50–75%) were idiotype-negative. The fact that not all B cells in the H-2-d/d Tg mice bear the idiotype is to be expected on the basis of earlier studies of allelic exclusion in immunoglobulin-transgenic mice (reviewed in ref. 16). Interestingly, almost all H-2-d/d Tg bone-marrow IgM^+ B cells were also idiotype-positive, indicating that rare variant B cells may be strongly selected in peripheral lymphoid organs. The idiotype-negative B cells in Tol 1 mice could be the result of somatic mutation of transgenes, or of the association of the Tg immunoglobulin heavy or light chains with light or heavy chains encoded by endogenous genes.

Our conclusion that deletion is taking place in H-2-d/k Tg mice rests on the following data. The peripheral B cells in these mice (as defined by the marker B220) all bear high concentrations of surface IgM but no detectable idiotype, and their frequency is reduced by ~50% compared with H-2-d/d Tg control littermates. Furthermore, in the bone marrow there is a striking difference in surface IgM levels between H-2-d/d Tg and H-2-d/k Tg mice, indicating that capping or down-regulation of immunoglobulin is occurring in the latter. If idiotype-producing B cells populate the spleen in H-2-d/k Tg mice, the staining patterns of the bone marrow and spleen cells of these mice would be expected to be similar. These results show that there must be a significant reduction in the number of id-producing B cells in the spleen and lymph nodes of H-2-d/k Tg mice, but they cannot exclude the possibility that a small number of such cells are spared. If residual id-positive B cells exist, they could well be in an activated state (having presumably encountered antigen) and might contribute to the levels of transgene-encoded mRNA observed in H-2-d/k spleen.

The expression of $H\text{-}2D^k$ and K^k on the B cells of H-2-d/k Tg mice is probably not required for clonal deletion. This conclusion is based on radiated bone-marrow chimaeras of the type $H\text{-}2^dTg \rightarrow H\text{-}2^d \times H\text{-}2^k$, indicating that the same bone marrow inoculum can give rise to id-positive B cells in an $H\text{-}2^d$ environment, but not in an $H\text{-}2^d \times H\text{-}2^k$ environment (data not shown). It is possible that in some cases the interaction of antigen receptor and antigen in the cell (perhaps in the endoplasmic reticulum) could permit specific signal transduction leading to tolerance; this might be an important mechanism for eliminating a wide variety of anti-self specificities and even play a part in T-cell tolerance.

In contrast to our results, a recent report[17] claims that functional inactivation of autoreactive B cells occurs without physical deletion in mice transgenic for anti-hen egg lysozyme. As these authors point out, the lack of clonal deletion in their system may be the result of the low concentration or univalent form of the autoantigen (transgene-encoded hen-egg lysozyme)

Table 1 Serum concentrations of immunoglobulins in (BALB/c × BALB/k) × Tol 1 mice

	IgM $\mu g\ ml^{-1}$	3-83 Idiotype $\mu g\ ml^{-1}$	Number of animals tested
Non-transgenic	846 ± 301*	<0.3 (<0.035%)†	13
H-2 d/d transgenic	151 ± 42	93 ± 34 (61.6%)	7
H-2 d/k transgenic	40 ± 21	<0.3 (<0.75%)	6

Sera were taken 22 days after birth. IgG titres were uniformly high in all mice and may have been of maternal origin. Radioimmunoassay was performed as follows: polyvinylchloride wells were coated overnight at 4 °C with 50 μl per well of PBS containing 5 μg ml^{-1} purified 54.1 (rat anti-idiotype), m41 (rat anti-μ) or YA2 (rat anti-IgG). Wells were washed 3 times and then incubated for 30 min with PBS, 1% bovine serum albumin, 0.02% sodium azide (buffer A). Various concentrations of serum or control antibodies diluted in buffer A were applied to the wells and incubated for 3 hours at 4 °C. After washing, 2.5 ng ^{125}I-labelled 33-18-12 (rat anti-mouse kappa) diluted in buffer A were applied to the wells for two hours at 4 °C. The wells were extensively washed with PBS and then with tap water and the bound radioactivity was measured in a gamma counter. As a positive control and to calibrate the IgM and idiotype assays, Cos lin D1 monoclonal antibody was used. This antibody is derived from the supernatant of Sp2/0 myeloma cells transfected with a cosmid containing the DNA fragments shown in Fig. 1 (ref. 14). For segregation analysis of transgenes and MHC genes, data were obtained from Southern blots using *Bam*HI-digested tail DNA. A number of hybridization probes, including pJ11 (see Fig. 1), could unambiguously distinguish Tg from non-Tg individuals. The complementary DNA probe p2IIa (ref. 20), which hybridizes to many class I genes, was used to distinguish H-2-d/k and H-2-d/d mice on the basis of three different H-2^k-specific fragment length polymorphisms.

* Errors listed are ±1 standard deviation.

† Per cent IgM that is of the 3-83 idiotype.

used. It may be that these B cells do not encounter the physiologically relevant form or concentration of hen-egg lysozyme until they are relatively mature, and hence must be dealt with in a different way, for example by suppression. It should also be pointed out that in the presence of antigen the numbers of hen-egg lysozyme-specific B cells in the transgenic mice are lower than in the controls lacking antigen, suggesting that either there is deletion of part of the clone (perhaps a particular B-cell subset) or that the lifespan of the entire clone is reduced. The MHC class I antigens used in our system are cell-surface proteins that could probably permit extensive cross-linking of immunoglobulin receptors on reactive B cells arising in the bone marrow. A signal at this developmental stage might lead directly to B-cell inactivation and death.

The *in vivo* deletion of self-reactive B cells agrees with recent experiments indicating that T cells bearing receptors for self-MHC antigens are also physically deleted[18]. Thus our results would support the hypothesis that B cells are made tolerant by antigen in the absence of antigen-reactive T cells[19]. Our Tg mice should allow us to explore more carefully the cellular and biochemical events leading to tolerance in the B-cell compartment.

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Spatial and temporal expression of the retinoic acid receptor in the regenerating amphibian limb

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Retinoic acid is known to have dramatic effects on vertebrate limb pattern in development and regeneration, supporting a model in which a gradient of retinoic acid serves as a morphogen to differentially supply positional information to a developing limb. The discovery of a retinoic acid receptor (RAR)[1,2] and its homology to the steroid and thyroid hormone receptors[3] provided a potential molecular mechanism for limb morphogenesis[4]. One prediction of this model is that the receptor must be expressed in the developing and regenerating limb anlage. We investigated the expression of the RAR in the adult newt, *Notophthalmus viridescens*, whose amputated limbs are capable of regenerating and upon which retinoic acid can act to alter pattern. We report the cloning of cDNAs encoding a functional newt RAR and the localization of high and uniform levels of RAR mRNA specifically in the regenerating cells that control limb pattern. These results indicate that the morphogenic field is established through differential activation of pre-existing retinoic acid receptors rather than differential expression of the RAR gene.

To confirm the existence of a RAR in the regenerating newt limb, a λgt10 cDNA library made from early bud stage, proximal hindlimb blastema poly(A)$^+$ RNA was screened with a human RARα cDNA[1] as probe. Two positive clones, designated λNB6 and λNB8, were isolated and their *Eco*R1 inserts subcloned in a pGEM4 plasmid (Fig. 1*a*). The composite nucleotide and predicted amino-acid sequences of this putative newt RAR cDNA are shown in Fig. 1*b*. In humans, two distinct RAR gene products hRARα[1,2] and hRARβ[5,6] have been identified. The newt protein is highly related to both human receptors with greater apparent similarity to the β-form, especially in the N-terminal region.

To test whether the newt RAR is a functional retinoic acid receptor, the expression plasmid RSnRAR was cotransfected in

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