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Peripheral deletion of self-reactive B cells

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Abstract

B lymphocytes are key participants in the immune response because of their specificity, their ability to take up and present antigens to T cells, and their capacity to differentiate into antibody-secreting cells. To limit reactivity to self antigens, autospecific B cells can be functionally inactivated or deleted[1-4]. Developing B cells that react with membrane antigens expressed in the bone marrow are deleted from the peripheral lymphocyte pool[4-6]. It is important to ascertain the fate of B cells that recognize membrane autoantigens expressed exclusively on peripheral tisues because B cells in the peripheral lymphoid organs are phenotypically and functionally distinct from bone-marrow B cells[7-9]. Here we show that in immunoglobulin-transgenic mice, B cells specific for major histocompatibility complex class I antigen can be deleted if they encounter membrane-bound antigen at a post-bone-marrow stage of development. This deletion may be necessary to prevent organ-specific autoimmunity.

To test for peripheral deletion double-transgenic (Dbl-Tg) mice were produced by mating mice bearing the *MT-K*^{b} transgene, which targets expression of the membrane major histocompatibility complex (MHC) class I antigen K^b to the liver[10], with 3-83$\mu\delta$ mice (Ig-Tg), which are transgenic for heavy and light chain immunoglobulin genes encoding anti-K^b antibody (see legend to Fig. 1). No messenger RNA or surface-protein expression of the K^b is detectable in lymphoid tissues of MT-K^b mice[10]. Both the Ig-Tg and MT-K^b transgenics were bred as hemizygous traits and (Ig-Tg × MT-K^b)F$_1$ littermates were analysed.

Double-transgenic Ig-Tg/MT-K^b mice deleted peripheral B cells. In Dbl-Tg lymph nodes there were >25-fold fewer cells bearing IgM(sIgM$^+$-cells) compared with Ig-Tg or non-Tg controls and there were no detectable 3-83 idiotype-positive cells (Fig. 2*a*; Table 1). This lack of IgM$^+$ B cells in Dbl-Tg lymph nodes was not the result of sIgM downregulation because there was a similar >25-fold decrease in cells bearing the B-cell surface marker B220 (CD45R) (Table 1) and because >95% of the remaining cells were T cells bearing the $\alpha\beta$ receptor ($\alpha\beta$-TCR$^+$ T cells; data not shown). In Ig-Tg mice, >95% of bone marrow, spleen and lymph node B cells bore the 3-83 idiotype and these cells expressed IgM and IgD at levels as high as normal (Non-Tg) littermates (Fig. 2). In contrast to the deletion of idiotype-positive peripheral B cells, no deletion was evident in the bone marrows of Dbl-Tg

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mice, which had as many $sIgM^+$/$idiotype^+$ B cells as Ig-Tg littermates (Fig. 2*a*), as would be expected if antigen was only encountered in the periphery. This is clearly different from 3-83 mice bearing K^b or K^k on the whole body, in which cells bearing a high density of sIgM are greatly depleted in the bone marrow (refs 4-6, and our unpublished results). Furthermore, the deletion is probably not the result of secreted or shed soluble K^b because of the tissue specificity of the deletion and because up to 200 ng ml^{-1} of the high-affinity ligand K^k fails to tolerize 3-83 μ cells *in vivo*[6].

Serum 3-83 idiotype levels were >80-fold lower in Dbl-Tg compared with Ig-Tg mice, as would be predicted if autoreactive B cells were eliminated (Table 1). Interestingly, the bulk of the spontaneously secreted IgM in these mice lacked the 3-83 idiotype and was present at a similar concentration in Dbl-Tg and Ig-Tg mice. Thus variant B cells that fail to produce the transgene-encoded idiotype are responsible for most of the circulating IgM in these mice.

Consistent with the interpretation that the autoreactive B cells are deleted, transgene 3-83κ mRNA levels paralleled the levels of idotype-positive B cells in the lymph nodes, spleen and bone marrow of Ig-Tg and Dbl-Tg mice (Fig. 3). In addition, 3-83κ mRNA levels in the livers of Dbl-Tg mice were not elevated compared with Ig-Tg mice, indicating that there was no accumulation of autoreactive B cells in the antigen-bearing organs (Fig. 3*a, b*). This conclusion was also supported by histological examination of liver sections (not shown).

The Dbl-Tg mice allowed us to test *in vivo* the notion that expression of IgD by B cells renders them impervious to tolerance induction[11,12]. The deleted cells in Dbl-Tg mice probably expressed IgD at the time they encountered antigen because IgD was expressed on a large fraction of the detectable B cells, including about half of the Dbl-Tg bone marrow B cells, which should not yet have encountered antigen (Fig. 2*b*). The absence of B cells in the lymph nodes of the Dbl-Tg mice indicates that these mice had a greatly depleted recirculating B-cell pool, and that most of the B cells present in the bone marrows and spleens of Dbl-Tg mice were probably newly formed. The remaining B cells in the spleens of Dbl-Tg mice had both IgD and idiotype. It is possible that these cells were generated by lymphopoiesis in the spleen or were in transit from the bone marrow and had not yet encountered K^b. In any case, there was a clear quantitative diminution of autoreactive cells that occurred outside the primary haematopoietic organ for B cells, the bone marrow. In addition, adoptive transfer of Ig-Tg lymph node and spleen B cells into 750-roentgen-irradiated K^b-bearing mice resulted in deletion (D.M.R. and D.N., unpublished results). These data are consistent with reports demonstrating that certain B-cell lymphomas undergo anti-Ig-κ-induced apoptosis *in vitro* whether or not sIgD is co-ligated with sIgM (refs 13, 14). On the other hand, IgD expression was probably not necessary for the observed deletion, because IgM-only 3-83 transgenic mice also delete autoreactive B cells in crosses with MT-K^b (ref. 6, and our unpublished data).

These data demonstrate B-cell clonal deletion to membrane autoantigens expressed in the periphery. They also indicate that B-cell deletion is unlikely to be mediated by a specialized antigen-presenting cell, such as a ‘veto’ cell[15], because in MT-K^b mice the K^b antigen is only detectable on the surfaces of hepatocytes, exocrine pancreatic cells and kidney tubules[10]. On the other hand, we do not believe that all peripheral membrane autoantigens induce B-cell deletion as double-transgenic mice expressing K^b under the control of a keratin promoter fail to delete idotype-positive B cells completely (D.N., B. Arnold and G. Hammerling, unpublished results). It is not yet clear whether K^b antigen density, location, availability, or timing of expression determine the differences between the MT-K^b/3-83$\mu\delta$ and keratin-K^b/3-83$\mu\delta$ Dbl-Tg mice.

Tolerance to many self proteins may rely on inactivation or deletion of antigen-specific T cells, B cells, or both. Because in some cases B cells are the limiting antigen-presenting cells for class II-restricted T-cell responses[16,17], deletion of autoreactive B cells should hinder both autoantibody formation and the presentation of autoantigens to class II-restricted T cells. Deletion of B cells in the periphery may be important in the prevention of tissue-specific autoimminue disease.

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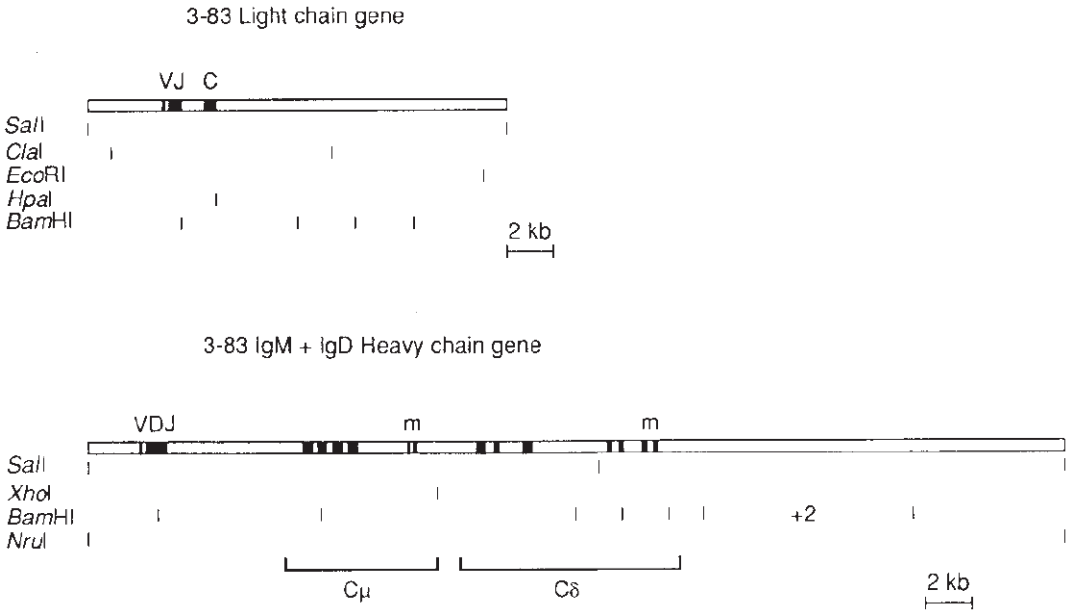

FIG. 1.

Maps of the DNA fragments used to generate 3-83$\mu\delta$ transgenic mice. Dark regions represent exons.

METHODS. Mice were produced using functional rearranged heavy- and light-chain genes encoding the 3-83 antibody. This antibody binds with moderate affinity to K^k, with weak affinity to K^b, and fails to bind to H–2^d cells[22]. Transgenic mice encoding the IgM form of 3-83 have been described[4-6]. Self tolerance to K^k and K^b in the IgM-only 3-83 mice is mediated by deletion in H–2^d transgenic → H–2^d × H–2^k or H–2^d transgenic → H–2^d × H–2^b bone marrow chimaeras[5,6]. To generate the 3-83 IgM plus IgD heavy-chain fragment, the 3-83μ construct used previously[4] was extended to include the complete $C\delta$ genomic locus. The 3-83μ insert was liberated from its vector by partial digestion with *Eco*R1 and cloned into the *Eco*R1 site of λEMBL3 to generate λ241. A cosmid clone spanning the (DBA/2-derived) Ig-Cμ and Ig-Cδ regions was then isolated from a genomic library of the T-cell hybrid BDF1 16 (ref. 23), linearized with *Xho*I, which cuts at the site between $C\mu$ and $C\delta$, and ligated together with the *VDJ/Cμ*-containing *Sal*I/*Xho*I fragment of λ 241 and with *Sal*I-digested pNNL cosmid vector[24], resulting in a cosmid whose restriction map in the $C\mu/C\delta$ region corresponds to the natural locus. The 42-kilobase (kb) insert was liberated from all but ~200 base pairs (bp) of the vector by *Nru*I digestion. Light- and heavy-chain gene fragments were isolated and transgenic mice produced as described[4]. Southern blotting and segregation analysis indicated that the 3-83$\mu\delta$ line has ~3–5 copies of the light- and heavy-chain genes co-integrated at a single chromosomal locus. m, Transmembrane exons.

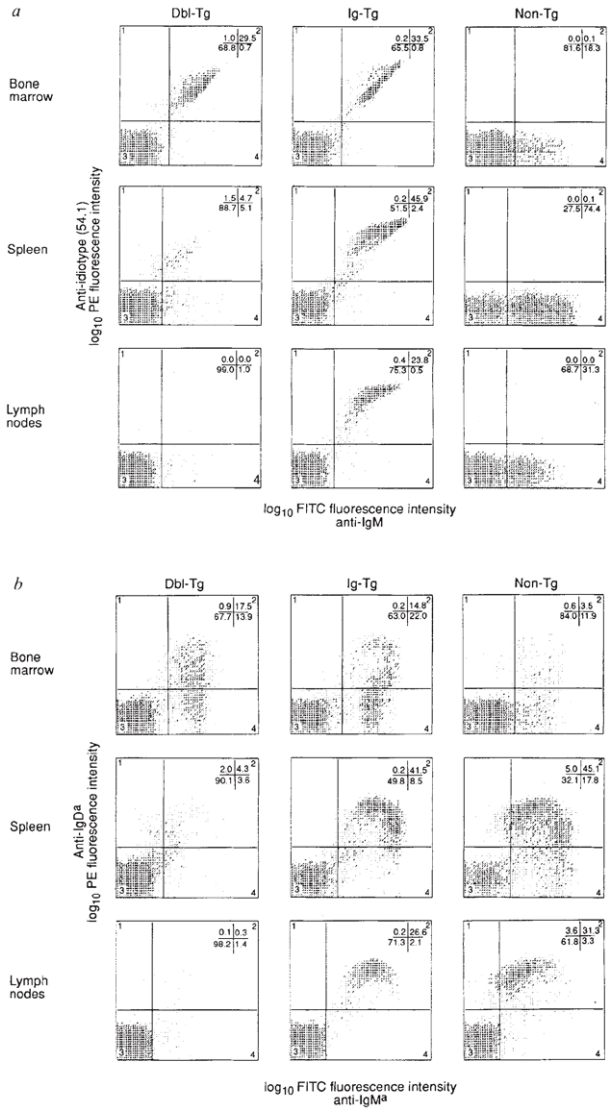

FIG. 2.

Elimination of autoreactive B cells in spleen and lymph nodes, but not in the bone marrow, of an Ig-Tg mouse bearing peripherally expressed antigen. Cells from the organs of 5-week-old transgenic and normal littermates were analysed by two-colour flow cytometry. *a*, Staining with anti-idiotype and anti-IgM. Idiotype was detected with the 3-83 clonotype-specific rat monoclonal antibody 54.1, which recognizes an epitope created by the pairing of 3-83 heavy and light chains[20]. *b*, Staining with anti-IgD and anti-IgM. Dbl-Tg, double transgenic (3-83$\mu\gamma$/MT-K^b); Ig-Tg, immunoglobulin transgenic; Non-Tg, non-transgenic. Cells were prepared as before and stained with monoclonal antibodies: anti-3-83 clonotype, 54.1/biotin; anti-IgM, DS1/FITC (ref. 25); anti-IgD^a, AMS 9.1/biotin (Pharmingen)[26]. Biotin-conjugated antibodies were revealed with phycoerythrin (PE)–streptavidin stain (Becton–Dickinson). Purification and fluorochrome conjugation of the antibodies was as described[27]. Flow cytometry analysis was on a Profile (Coulter) machine and data are presented using its density plot function. The percentages of cells in each quadrant, rounded to the nearest 0.1%, are indicated in the upper right corner of each plot. Data for bone marrow cells are gated by side scatter to exclude the predominant myeloid cell population from analysis. All mice used in this experiment were H–2^d and Igh-C^a at the endogenous

loci. The genetic background of the mice is ~75% BALB/cJ, 6% B10.D2, 6% C57b16/J, 6% SJL/J, 6% DBA/2. The transgenic havy chain μ and δ allotypes are type *a*. The results shown are representative of 4 experiments (Table 1). MT-K^b mice were indistinguishable from Non-Tg mice in these assays (not shown).

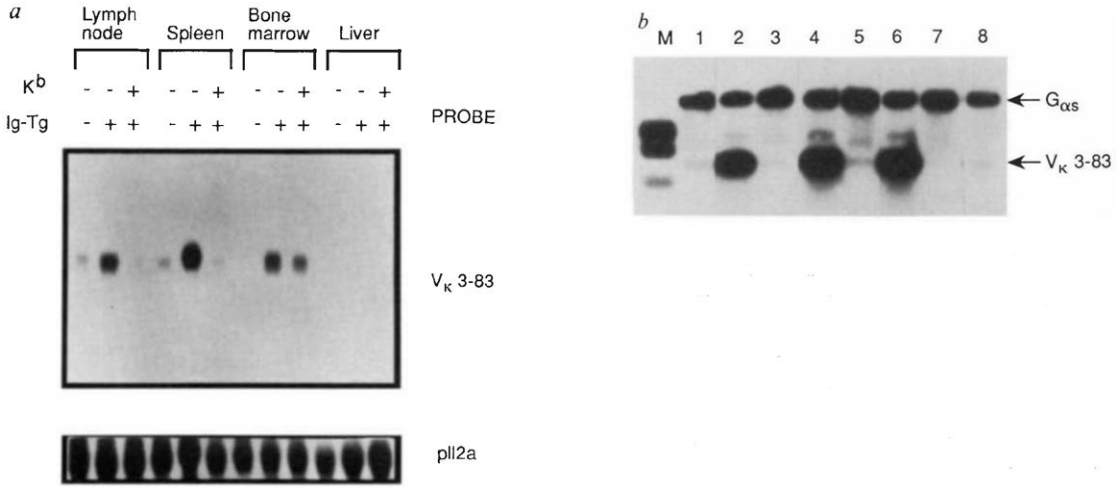

FIG. 3.

RNA analysis of transgenic and non-transgenic mice. *a*, Northern blot analysis shows reduction in transgenic κ-light chain expression in spleen and lymph nodes of Dbl-Tg mice. After hybridization with V_κ3-83 probe[4] and decay of the radioactive signal, the filter was hybridized with the MHC class 1-specific pll2a probe[28]. *b*, Polymerase chain reaction (PCR) detection of V_κ3-83 RNA in the livers of peripherally deleting mice. Lanes 1, 3, 5 and 7, liver; lanes 2, 4, 6 and 8, bone marrow. Lanes 1 and 2, mouse 2080 (Dbl-Tg); lanes 3 and 4, mouse 2081 (Dbl-Tg); lanes 5 and 6, mouse 2082 (Ig–Tg); lanes 7 and 8, Non-Tg littermate. Complementary DNA was synthesized and amplified with 15 cycles of PCR, run on a 1.2% agarose gel, transferred to a Zetaprobe membrane (BioRad) and hybridized with probes recognizing gene transcripts of 3-83 V_κ or the GTP-binding protein $G_{\alpha s}$ (ref. 29). Lane M, marker DNA fragments of 587, 434 and 267 bp.

METHODS. RNA preparation and northern blotting were as described[4]. 5μg RNA was loaded per lane in the 1% agarose formaldehyde gel. RNA samples were reverse-transcribed using a commercial kit (Superscript, BRL). PCR reactions were in a final volume of 30 μl and contained 1 μl reverse transcription reaction, 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 20 μg ml^{-1} gelatin, 0.3 mM each of dATP, dCTP, dGTP and dTTP, 1 μM of each oligonucleotide primer, and 1 U of *Taq* polymerase (Perkin–Elmer Cetus). Each cycle consisted of 30 s at 94 °C, 30 s at 62 °C and 1.5 min at 72 °C in a thermal cycler (Ericomp). The appropriate cDNA clones were used as hybridization probes after labelling with [α-^{32}P)dCTP. PCR primers were: 5′Vκ3-83 (79-101): 5′-CAGCTTCCTGCTAATCAGTGCC-3′; 3′Jκ2 (412-431): 5′-TGGTCCCCCCTCCGAACGTG-3′; $G_{\alpha s}$ sense (309-336): 5′-ATTGAAACCATTGTGGCCGCCATGAGC-3′; $G_{\alpha s}$ antisense (1,104-1,128): 5′-GAAGACACGGCGGATGTTCTCAGTGTC-3′.

TABLE 1

Ability of peripherally expressed K^b antigen to induce B-cell tolerance as assayed by reduction in the amount of serum IgM idiotype and decrease in the number of peripheral B lymphocytes

Serum immunoglobulin*

n	Type	IgM (μg ml^{-1})	3-83 idiotype (μg ml^{-1})
2	Non-Tg	360 ± 90	<0.4
5	Ig–Tg	98 ± 47	34 ± 16
5	Dbl-Tg	103 ± 36	<0.4

Peripheral B cells†

		Total nucleated cells (×10^{-6})		B220 + cells (×10^{-6})		% IgM positive		% B220 positive	
n	Type	Spleen	Lymph nodes‡	Spleen	Lymp nodes	Spleen	Lymph nodes	Spleen	Lymph nodes
3	Non-Tg	109 ± 16	67 ± 15	62	20	53.9 ± 14.6	28.2 ± 6.0	56.6 ± 12.1	30.0 ± 4.5
4	Ig-Tg	105 ± 38	81 ± 21	43	20	44.4 ± 7.5	24.9 ± 5.1	41.1 ± 5.6	25.0 ± 3.6
5	Dbl-Tg	77 ± 21	62 ± 20	12	**0.7**	9.1 ± 2.5	**0.8 ± 0.4**	11.4 ± 4.1	**1.2 ± 0.6**

Immunoglobulin concentrations were measured using standard enzyme-linked immunosorbent assay (ELISA) techniques[18]. Polyvinylchloride plastic microtitre plates were coated with rat monoclonal antibodies specific for IgM (Ak15; ref. 19) or 3-83 idiotype (54.1). After washing and blocking, sera (diluted in PBS supplemented with 1% BSA) were incubated for 3 h at 25 °C. Bound immunoglobulin was detected by biotinylated rat anti-mouse IgM (Ak2; ref. 19) and developed using streptavidin–peroxidase (Sigma) followed by colorimetric substrate. Absorbance was measured on a BioRad model 2225 ELISA plate reader. Data were analysed using the Microplate Manager program. Standard curves for the assay were generated using a supernatant from Cos lin D1, an Sp2/0 myeloma transfected with genes encoding κ and μ chains of 3–83 (ref. 20). Immunofluorescence analysis was performed as described for Fig. 1. Anti-B220 (CD45R) antibody was FITC-RA3-3A1 (ref. 21).

*All data are presented as mean ± s.d. Mice were 6–8 weeks old.

†All data are presented as mean ± s.d. Mice were 4–8 weeks old. Data are from 4 independent experiments done on different days. Each experiment included one Ig-Tg mouse, at least one Dbl-Tg littermate and, in all but one experiment, a non-Tg littermate.

‡Cells were pooled from the following lymph nodes: superficial inguinal, brachial, axillary, superficial cervical, mesenteric and popliteal.