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Clonal deletion of autoreactive B lymphocytes in bone marrow chimeras

(immunological tolerance/transgenic mice/histocompatibility complex)

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ABSTRACT **To study the fate of developing B cells in the presence and absence of the autoantigens to which they react, chimeric mice were constructed by injecting bone marrow cells from mice transgenic for rearranged immunoglobulin genes encoding an anti-H-2K^k antibody into irradiated recipients that did or did not express the H-2K^k antigen. In the presence of H-2K^k, the anti-H-2K^k-specific B cells were deleted from the spleen and lymph nodes, whereas in its absence, anti-H-2K^k cells were abundant. B cells bearing a low level of membrane immunoglobulin with the anti-H-2K^k idiotype were found in the bone marrows of *H-2K^k* recipients, suggesting that clonal deletion of autoreactive cells was occurring in the pre-B-cell to B-cell transitional stage of B-cell development.**

Self-tolerance is a basic property of the immune systems of vertebrates. The mechanism by which tolerance is thought to be achieved is the selective removal or inactivation of self-reactive lymphocytes, whose clonally distributed antigen receptors confer specificity (1). Until recently, tolerance in the B-lymphocyte compartment has been studied primarily by measuring functional parameters of antibody secretion and plaque-forming cell production. These experiments have in many cases supported the idea that tolerance exists at the B-cell level (reviewed in ref. 2) but could not unambiguously distinguish among the possible mechanisms by which it occurs: clonal deletion, suppression, or "anergy," in part because of the heterogeneity and low frequency of antigen-specific B cells. The generation of transgenic mice with functional immunoglobulin genes that encode antibody molecules (and therefore B-cell receptors) of known specificity (3) allows the study of a large clone of B cells with a predetermined antigenic specificity, thereby making it technically possible to determine the fate of autoreactive B cells *in vivo* (4, 5). These mice allow one to ask the question: Do autoreactive B cells persist or are they physically eliminated?

Two B-cell tolerance experiments have been carried out with Ig transgenic mice (4, 5), and the conclusions from the two studies were different, underscoring the difficulty in interpreting previous results obtained with less direct approaches. In the first experiment, transgenic mice bearing anti-hen egg lysozyme-encoding Ig genes contained comparable numbers of hen egg lysozyme-specific B cells in the peripheral lymphoid organs, whether the tolerizing antigen was present or not. In the presence of (auto)antigen, these cells were nonfunctional in short-term immunization assays and bore subnormal levels of surface IgM (4). In contrast, our previous results, measuring tolerance to major histocompatibility complex (MHC) class I proteins in anti-H-2^k transgenic mice, indicated that the self-specific B cells were deleted (5). This study was done by comparing B cells in F_1 transgenic mice that either did or did not bear the H-2^k antigens to which the transgenic antibody reacts (H-2K^k and H-2D^k).

A potential difficulty with studying tolerance to H-2^k in our system with genetic crosses was that, because the B cells in the tolerant animals also bore H-2 class I antigens, it was not clear whether it was necessary for the H-2^k antigens to be coexpressed on the transgenic B cells themselves to achieve tolerance. In this report we describe the tolerance phenotype of irradiation bone marrow chimeras in which anti-H-2^k transgenic B cells that lack the H-2^k antigens develop in hosts that bear or do not bear H-2^k antigens. The results support our previous conclusion that the tolerance of B cells specific for class I MHC antigens is mediated by clonal deletion.

MATERIALS AND METHODS

Antibodies and Immunofluorescence. The following rat IgG monoclonal antibodies were used as hybridoma culture supernatants in immunofluorescence: M41 (anti-mouse IgM) (6), R33-24-12 (allotype-restricted anti-mouse IgM) (6), 54.1 (anti-3-83 idiotype) (5), BaG1 (anti-lipopolysaccharide, a negative control; ref. 21). Binding of rat antibodies was revealed with fluorescein isothiocyanate (FITC)-labeled F(ab')$_2$ mouse anti-rat polyclonal antibody (Jackson ImmunoResearch). Hybridoma RA3-3A1 (IgM rat anti-mouse CD-45R "B220") (7) was obtained from the American Type Tissue Culture Collection; its antibody was affinity-purified from hybridoma supernatant on protein A-Sepharose (Sigma), was fluoresceinated (8), and was used at an empirically determined optimal amount of 0.1 μg per 10^6 cells in a final volume of 100 μl. Anti-I-A^d and anti-I-A^k monoclonal alloantibodies (Becton Dickinson) were used at the concentration recommended by the manufacturer and developed with a specific second-step antibody, FITC-goat anti-mouse IgG2a or FITC-goat anti-mouse IgG2b (Southern Biotechnology Associates, Birmingham, AL), respectively. Phycoerythrin-labeled rat anti-mouse immunoglobulin κ light chain was from Becton Dickinson and used according to the manufacturer's instructions. The mouse IgG2a anti-H-2K^{k}D^k antibody 3-83 (9) was used as hybridoma supernatant and developed with FITC-goat anti-mouse IgG2a (Southern Biotechnology Associates).

Cell preparation, staining protocol, and flow cytometry analysis were as described (5) except that both one- and two-color fluorescence were analyzed on a FACScan flow cytometer (Becton Dickinson).

Hybridomas M41 and 33-24-12 were provided by Anton Rolink, and BaG1 supernatant was the gift of Gerd Pluschke.

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Abbreviations: Tg, transgenic; MHC, major histocompatibility complex; FITC, fluorescein isothiocyanate; BSS, balanced salt solution.

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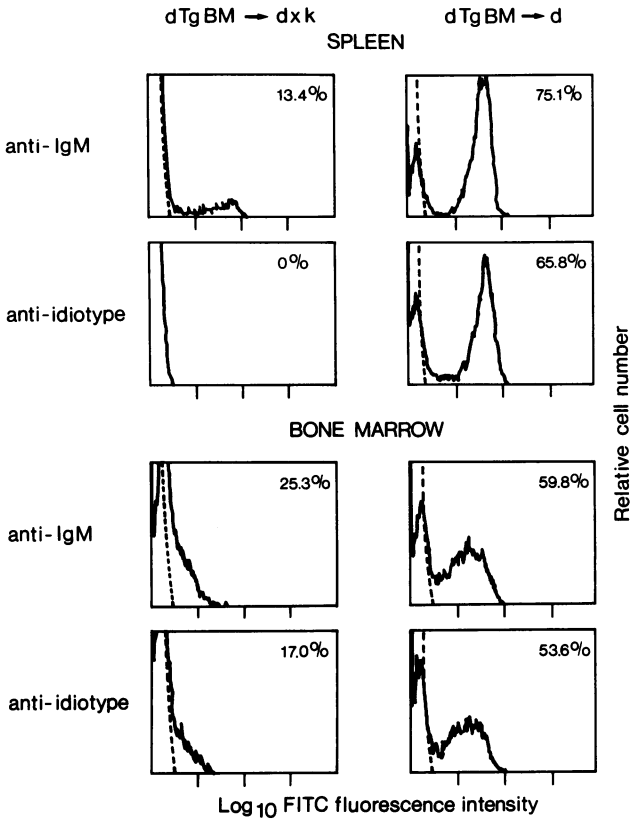

FIG. 1. Immunofluorescence analysis of bone marrow (BM) chimera B cells. Data are from experiment 1A (see Table 2). Cells were stained with the indicated rat IgG monoclonal antibodies developed with a second-step FITC-mouse $F(ab')_2$ anti-rat IgG antibody. Solid lines represent the staining pattern with the indicated antibodies; dashed lines show background staining for each cell type when using the control rat IgG antibody BaG1 in the first staining step. Bone marrow data were gated to exclude from analysis the ≈80% of cells that were of the larger, predominantly myeloid type.

Mice. BALB/cJ, A/J, C.AL-20, B10.D2/oSnJ, and B10.A and F_1 hybrids of these strains were obtained from the Basel Institute for Immunology's breeding facility (Kaiseraugst, Switzerland). The transgenic (Tg) line Tol 1 (5), established by the microinjection of DBA/2 × B10.D2 F_2 zygotes with genes encoding the IgM form of the IgG2a anti-H-$2K^kD^k$ antibody 3-83 (9), was bred and maintained at the Basel Institute for Immunology.

Bone Marrow Donors. Mice expressing microinjected anti-*H-2*^{k} genes (line Tol 1) were produced as described (5) and back-crossed to BALB/cJ mice once (experiments 1A–1D) or twice (experiments 2A and 2B). In experiments 2A and 2B, both Tg and non-Tg (normal) littermates generated from the same cross were used as donors, whereas in experiments 1A–1D, only Tg donors were used. Mice were checked for the presence or absence of the transgenes and for *H-2* haplotype by using Southern blot analysis as described (5). All donors were *H-2*^{d} and 8–12 weeks old.

Irradiated Recipients. Starting 1 week before bone marrow transfer, mice were maintained on acidified water containing 1 mM neomycin sulfate. Mice were exposed to 900 rads of γ-irradiation from a ^{137}Cs source, immediately injected i.v. with 5×10^6 T cell-depleted bone marrow cells, and housed in isolators for the duration of the experiment.

Preparation of Bone Marrow Suspensions. Femurs were removed sterilely, ground in a mortar containing 10 ml of ice-cold sterile balanced salt solution (BSS), and filtered through cotton wool to remove bone and debris. The cells were depleted of erythrocytes by using Gey's solution, washed twice in BSS, and incubated for 30 min on ice in BSS containing 10 μg of purified T24 per ml, which is a pan-T-cell-specific rat anti-mouse monoclonal antibody that was a gift from U. Staerz. Cells were then centrifuged, resuspended in rabbit complement (Cedarlane Laboratories, Hornby, ON, Canada), diluted to 10% in BSS, and incubated at 37°C for 45 min. Cells were then centrifuged, resuspended in BSS, refiltered through cotton wool, washed twice in BSS, and counted. Viable cell concentration was adjusted to 5×10^7 per ml in BSS, and 0.1 ml was injected i.v. per irradiated recipient. Aliquots of cells taken before and after antibody + complement treatment were analyzed for the presence of T cells by using immunofluorescence to determine the efficiency of the treatment. In the experiments described, T-cell contamination was reduced to <0.5%.

Table 1. Summary of bone marrow (BM) chimera data

Exp.	BM source	Recipient	Weeks after reconstitution	Viable cells recovered × 10^{-6}		Idiotype$^+$/IgM$^+$ (ratio × 100)	
				Spleen	BM	Spleen	BM
1A	*H-2*^{d} Tg	B10.A × BALB/c	7	24	51	0	67.2
	H-2^d Tg	B10.D2 × BALB/c	7	56	42	87.6	89.6
1B	*H-2*^{d} Tg	B10.A × BALB/c	7	20	10	0	42.1
	H-2^d Tg	B10.D2 × BALB/c	7	50	23	90.2	96.8
	—	Unirradiated control		49	34	0	0
1C	*H-2*^{d} Tg	B10.A × BALB/c	10	34	39	0	48.5
	H-2^d Tg	B10.D2 × BALB/c	10	30	29	72.8	87.4
	—	Unirradiated control		76	25	0	0
1D	*H-2*^{d} Tg	B10.A × BALB/c	10	24	13	0	73.3
	H-2^d Tg	B10.D2 × BALB/c	10	23	27	66.5	79.5
	—	Unirradiated control		87	27	0	0
2A	*H-2*^{d} Tg	C.AL-20 × A/J	4	76	16	0	5.7
	H-2^d Tg	C.AL-20	4	36	28	44.4	75.0
	H-2^d non-Tg	C.AL-20 × A/J	4	48	12	0	0
	H-2^d non-Tg	C.AL-20	4	44	27	0	0
	—	Unirradiated C.AL-20 × A/J		32	20	0	0
2B	*H-2*^{d} Tg	C.AL-20 × A/J	6	12	28	0	25.6
	H-2^d Tg	C.AL-20	6	16	27	47.6	83.6
	H-2^d non-Tg	C.AL-20 × A/J	6	40	16	0	0
	H-2^d non-Tg	C.AL-20	6	64	36	0	0
	—	Unirradiated C.AL-20 × A/J		43	24	0	0

RESULTS

Experimental Design. To directly compare the development of autoreactive B cells in the presence or absence of "self" antigen, sets of irradiation chimeras were constructed by i.v. injection of *H-2*^{d} Tg (or control non-Tg littermate) bone marrow suspensions into irradiated hosts that were either *H-2*^{d} or *H-2*^{d} × *H-2*^{a}. Mice with the *H-2*^{a} haplotype express H-2K^k, I-A^k, I-E^k, and H-2D^d MHC antigens; thus, the latter hosts bear the H-2K^k antigen to which the transgene-encoded antibody of the Tol 1 line reacts. The chimeras are designated "*d*Tg → *d* × *k*," "*d*Tg → *d*," "*d* → *d* × *k*," or "*d* → *d*" in the text to indicate the MHC type (*d* and *k* are alleles) at the *H-2K* locus and the type of bone marrow inoculum. To control for possible variations among B-cell progenitor populations, equal aliquots of the same bone marrow suspensions were injected into each type of host used in a given experiment. Reconstituted mice were analyzed 4–10 weeks later by immunofluorescence to determine whether B cells reactive with H-2K^k (as measured by binding with the 3-83-idiotype-specific antibody 54.1) were present in the bone marrow or peripheral lymphoid organs. In each tissue tested we also performed several controls to determine the extent of chimerism, the frequency of IgM-bearing cells, and the frequency of B220-bearing cells. In all chimeras, >90% of the B cells were donor-derived (see below).

Autospecific B Cells Are Found in the Bone Marrow But Not in the Lymph Nodes or Spleens of *d*Tg → *d* × *k* Chimeras. Fig. 1 shows histogram plots of immunofluorescence results typical of several experiments (see Tables 1–3): In *d*Tg → *d* mice, a high frequency of B cells, almost all of which were idiotype positive, was found in the spleen and bone marrow, whereas in the spleens of *d*Tg → *d* × *k* mice, no idiotype-positive cells were found, but a small population of idiotype-negative B cells bearing a normal density of surface IgM was apparent. Idiotype-positive cells were found in the bone marrows of *d*Tg → *d* × *k* mice, but they displayed a several-fold lower surface density of both idiotype and IgM compared to bone marrow cells from *d*Tg → *d* mice (Fig. 1).

These results strongly suggested that in the presence of H-2K^k antigen idiotype-bearing cells were selectively eliminated or excluded from peripheral lymphoid organs. Not only were idiotype-positive cells not detected in the spleens and lymph nodes of *d*Tg → *d* × *k* mice, but both the frequency and absolute numbers of B cells (as defined by expression of surface IgM, B220, or in some cases Ia) was reduced relative to *d*Tg → *H-2*^{d} controls, as would be expected if a net deletion occurred. In addition, the low-density cell surface expression

Table 2. Immunofluorescence analysis of bone marrow chimera tissues

Exp.	Group*	Tissue	Positive cells, %					
			IgM	Idiotype	B220	H-2^k	Ia^k	Ia^d
1A	*d*Tg → *d* × *k*	Spleen	13.4	0	28.4	8.6	0.5	23.2
	*d*Tg → *d*		75.1	65.8	79.7	0	0	74.9
	*d*Tg → *d* × *k*	Lymph nodes	11.2	0.1	13.6	12.6	0.3	ND
	*d*Tg → *d*		74.6	63.0	77.3	0	0	ND
	*d*Tg → *d* × *k*	BM (gated)†	25.3‡	17.0‡	68.4	0.2	0	ND
	*d*Tg → *d*		59.8	53.6	67.3	0	0	ND
1B	*d*Tg → *d* × *k*	Spleen	20.2	0	33.6	9.5	0.9	40.4
	*d*Tg → *d*		77.9	70.3	76.4	0	0	81.2
	Control *d* × *k*§		55.5	0	59.7	97.6	53.8	53.6
	*d*Tg → *d* × *k*	Lymph nodes	7.9	0	21.7	10.6	0.6	ND
	*d*Tg → *d*		66.0	50.0	68.1	0	0	ND
	Control *d* × *k*§		40.0	0	37.8	99.1	41.4	ND
	*d*Tg → *d* × *k*	BM (gated)†	19.0‡	8.0‡	72.9	0.6	0	ND
	*d*Tg → *d*		74.6	72.2	74.0	0	0	ND
	Control *d* × *k*§		36.7	0	74.7	89.9	58.2	ND
1C	*d*Tg → *d* × *k*	Spleen	16.7	0	19.8	ND	0.8	22.8
	*d*Tg → *d*		62.9	45.8	63.1	ND	0	65.7
	Control *d* × *k*§		57.9	0	52.8	ND	54.8	56.1
	*d*Tg → *d* × *k*	Lymph nodes	12.2	1.0	21.8	ND	0	ND
	*d*Tg → *d*		49.6	31.7	42.3	ND	0	ND
	Control *d* × *k*§		36.7	0	31.9	ND	34.7	36.5
	*d*Tg → *d* × *k*	BM (gated)†	23.3‡	11.3‡	82.0	ND	4.4	86.0
	*d*Tg → *d*		72.3	63.2	78.8	ND	0	80.9
	Control *d* × *k*§		29.7	0	76.5	ND	49.3	64.4
1D	*d*Tg → *d* × *k*	Spleen	21.9	0	29.5	ND	0.2	17.9
	*d*Tg → *d*		48.4	32.2	43.6	ND	0	39.7
	Control *d* × *k*§		59.2	0	59.0	ND	62.5	79.7
	*d*Tg → *d* × *k*	Lymph nodes	5.4	0	9.7	ND	0	19.4
	*d*Tg → *d*		17.7	11.3	18.6	ND	0	40.8
	Control *d* × *k*§		20.6	0	14.6	ND	17.8	58.3
	*d*Tg → *d* × *k*	BM (gated)†	40.0‡	29.3‡	92.2	ND	0	79.7
	*d*Tg → *d*		68.3	54.3	70.1	ND	0	58.3
	Control *d* × *k*§		32.7	0	85.2	ND	77.0	71.0

ND, not determined; BM, bone marrow.

*These recipients are *H-2*^{d} × *H-2*^{a}; the *d* × *k* refers to alleles at the *H-2K* locus. The Tg antibody binds to the H-2K^k molecule.

†Bone marrow cells were analyzed excluding electronically the larger, mostly myeloid cell types on the basis of their 90° light scatter characteristics.

‡Low density compared with the *d*Tg → *d* group.

§Unirradiated, uninjected recipient-type control.

Table 3. Immunofluorescence analysis of bone marrow chimera tissues

Exp.	Group	Tissue	Positive cells, % IgM* Total	Donor specific	Idiotype	B220
2A	dTg → $d \times k$	Spleen	2.7	2.3	0	4.3
	dTg → d		28.5	27.5	12.8	27.4
	$d \rightarrow d \times k$		51.2	56.2	0	51.6
	$d \rightarrow d$		60.3	61.4	0	56.0
	Control $d \times k$		57.1	1.0	0	54.0
	dTg → $d \times k$	BM (gated)†	7.4	5.3	0.3‡	66.7
	dTg → d		33.3	32.0	24.0	77.3
	$d \rightarrow d \times k$		30.5	34.8	0	84.5
	$d \rightarrow d$		27.0	29.5	0	80.0
	Control $d \times k$		31.0	0.1	0	79.5
2B	dTg → $d \times k$	Spleen	12.7	9.2	0	16.9
	dTg → d		53.9	53.0	38.9	51.8
	$d \rightarrow d \times k$		76.2	70.4	0	75.7
	$d \rightarrow d$		77.9	77.6	0	78.3
	Control $d \times k$		68.3	2.0	0	64.0
	dTg → $d \times k$	BM (gated)†	8.9	9.0	2.3‡	83.4
	dTg → d		37.2	35.9	30.0	75.8
	$d \rightarrow d \times k$		41.9	40.0	0	92.3
	$d \rightarrow d$		39.7	39.4	0	89.4
	Control $d \times k$		35.7	2.1	0	87.3

*Antibodies used were either the pan-IgM-specific M41 or 332412, which fails to see the recipient-type IgM.
†Bone marrow (BM) cells were analyzed excluding electronically the larger, mostly myeloid cell types on the basis of 90° light scatter.
‡Low density compared with the dTg → d group.

of idiotype and IgM in the bone marrow of dTg → $d \times k$ mice suggested the site of this deletion process was the bone marrow.

The allotype of the IgM on the splenic and bone marrow B cells was of the donor type (experiments 2A and 2B). Thus, the idiotype-negative B cells in dTg → $d \times k$ chimeras were variant donor-derived Tg cells that lacked the idiotypic determinant, possibly because of endogenous Ig gene rearrangement. This result underscores the fact that the deletional tolerance of autoreactive B cells that we observe is specific and spares nonautoreactive cells.

Lack of Idiotype-Bearing Cells in the Peripheral Lymphoid Organs of dTg → $d \times k$ Mice Was Not Due to Simple Cell-Surface Modulation of Anti-H-2k Antibody. The percentages of Ig^+, $B220^+$, and Ia^+ cells in the dTg → $d \times k$ spleens and lymph nodes were far lower than in the dTg → d controls (Tables 2 and 3), suggesting that B-cell numbers were reduced. Simple modulation or down-regulation of surface Ig should not have eliminated Ia on the B cells (10). On the other hand there was a consistent discrepancy between the percentages of IgM^+ and $B220^+$ cells in dTg → $d \times k$ spleens and lymph nodes, the $B220^+$ percentages being almost always higher (Tables 2 and 3). Two-color immunofluorescence analysis demonstrated two populations of $B220^+$ cells in the lymph nodes and spleens of many dTg → $d \times k$ mice: an Ig^+ population and an Ig^- population (Fig. 2). Compared to the Ig^+ cells, the surface Ig^- $B220^+$ cells had a distinctly lower density of B220 that is reminiscent of bone marrow pre-B-cells (compare Figs. 2 and 3). We believe that these were in fact pre-B-cells because they were seen in the lymph nodes and spleens of most dTg → $d \times k$ irradiation chimeras, and, shortly after reconstitution, in some dTg → d mice (data not shown) but not in $(d \times k)F_1$ Tg mice generated by breeding (5).

Abundance of pre-B-Cells and the Lack of B Cells in the Bone Marrows of dTg → $d \times k$ Chimeras. Fig. 3 shows results of

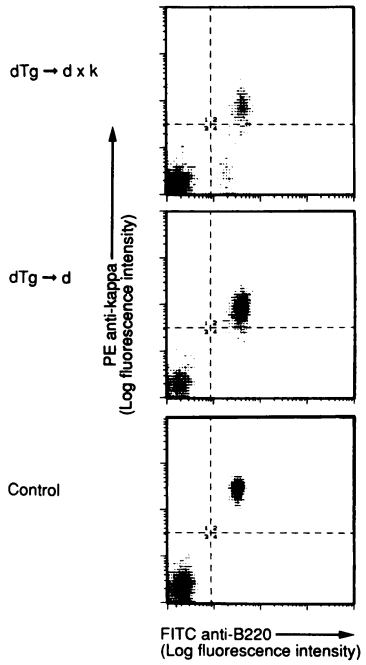

FIG. 2. Two-color immunofluorescence analysis of chimera and normal lymph node cells. (*Top*) Note that the $B220^+$ cells in the lower right quadrant have a clearly lower density of B220 than do the Ig^+ cells (in the upper right quadrant), and that such cells are absent from the cells analyzed in the lower two panels. These data were confirmed in many experiments, and double peaks were also seen when single-color analysis with anti-B220 was done (not shown). Data are from mice of experiment 1C (Table 2). Cells were incubated with phycoerythrin-conjugated anti-κ light chain antibody, washed, and incubated with FITC-conjugated anti-B220 (antibody RA3-3A1). The approximately 2-fold brighter fluoresence of the control cells with phycoerythrin-conjugated anti-κ chain antibody (*Bottom*) was not a reproducible finding and also was not seen in irradiated mice reconstituted with control bone marrow (data not shown). The graphic representation of the data was done by using the density plot function of the FACScan flow cytometer on data collected from 10,000 cells.

two-color flow cytometric analysis of chimera bone marrow cells stained with anti-B220 and rat anti-mouse Ig κ light chain antibodies that were typical of most such experiments. Fig. 3 *Bottom* shows the pattern of staining obtained with control normal bone marrow cells (gated to exclude dead cells and most myeloid cells from analysis). The cells fall into three categories: $B220^+$ Ig^+ B cells, $B220^+$ Ig^- pre-B-cells, and $B220^-$ Ig^- cells. Note that as the pre-B-cells express surface Ig and differentiate into B cells, they acquire a higher level of B220 (see also ref. 11).

While bone marrows of irradiation chimeras reconstituted with non-Tg bone marrow appeared essentially identical to the bone marrows of normal mice (data not shown), the dTg → d and dTg → $d \times k$ chimera bone marrow cells differed from them in several important respects (Fig. 3 *Top* and *Middle*). First, few dTg → $d \times k$ bone marrow cells had either a high density of surface Ig or B220, and the cells bearing an intermediate density of B220 bore low but clearly detectable amounts of surface Ig, as would be consistent with the results shown in Fig. 1. Second, in the dTg → d bone marrow cells, a relatively normal-looking B-cell population was present, but the intermediate-density $B220^+$ cells had levels of surface Ig almost as high as normal bone marrow or splenic B cells. Thus, in dTg → d mice, cells with a pre-B-cell phenotype were rare and B cells were abundant, whereas the opposite was true in dTg → $d \times k$ mice, where B cells were rare and pre-B-cells were abundant. The Ig^{high} $B220^{intermediate}$ cells in dTg → d bone marrow were likely the result of the premature expression of the transgenes in the pre-B-cell compartment

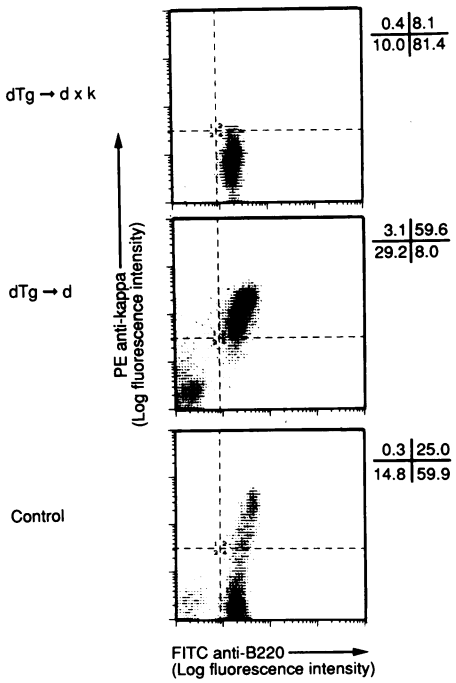

FIG. 3. Two-color immunofluorescence analysis of bone marrow cells taken from the same mice as in Fig. 2. Note the lack of Ig^+ (top right quadrant) or $B220^{high}$ (top right quadrant) cells in the dTg → $d \times k$ sample and the prominent Ig^+ $B220^{intermediate}$ cells (in the top right quadrant) in the dTg → d sample. Note also that the dTg → $d \times k$ bone marrow cells resembled the unusual Ig^+ $B220^-$ population in the lymph nodes of the same mice. Cells were stained and analyzed as in Fig. 2.

because such cells were found only in the bone marrow and not in the peripheral lymphoid organs. The existence of such cells tends to support the idea that the B220 level is independently regulated and is not linked to surface Ig expression. Thus, the lack of high-density $B220^+$ cells in dTg → $d \times k$ bone marrow implies that B-cell development was blocked in the majority of the cells.

DISCUSSION

The results of this study indicate that autoreactive B cells are clonally deleted. These data, obtained by using bone marrow chimeras to study the fate of autoreactive transgenic B cells, are quite similar to what we previously reported in F_1 crosses bearing H-2^k antigens (5), where B cells expressing the transgene-encoded Ig receptor specific for H-$2K^k$ were eliminated in mice bearing this antigen. The dTg → $d \times k$ chimeras had a reduced number of splenic B cells compared to chimeras lacking the H-$2K^k$ antigen, and they demonstrated an unusually low cell-surface expression of IgM on bone marrow B cells. The lack of the anti-H-2^k idiotype on the remaining peripheral B cells in dTg → $d \times k$ mice appears to have been the result of rearrangement and expression of endogenous Ig genes in these cells (D.N., unpublished data). In addition we found that both $(d \times k)F_1$ Tg and dTg → $d \times k$ bone marrows had greatly reduced numbers of cells bearing a high density of B220, which is normally a marker associated with Ig^+ cells.

No evidence was obtained to confirm the presence of "anergic" antigen-specific B cells in the spleens of tolerized mice as was seen in the hen egg lysozyme system (4). Although the lysozyme experiments reported used mice Tg for both IgM and IgD anti-lysozyme, these workers obtained similar results with an "IgM only" construct (22), arguing against the possibility that the absence of IgD anti-H-$2K^k$ on the surface of B cells in our mice accounts for the lack of anergy. Though not disproved, the original suggestion that IgD (whose function is unknown) plays a unique role in tolerance (12) lacks support (13). Our working hypothesis to explain our strikingly different result is that, because of its location on cell surfaces, the H-$2K^k$ antigen used in this study is better able to crosslink the surface Ig receptors of antigen-specific B cells [and thereby transmit a signal (14)] than the hen egg lysozyme used by Goodnow *et al.* (4), which was probably primarily in a monomeric soluble form. In this regard Pike *et al.* (15) have proposed, based on experiments with anti-IgM antibody *in vitro*, that a weak signal transmitted via the Ig receptor can lead to inactivation without deletion ("anergy") in immature B cells, whereas a strong signal may lead to deletion. We believe that the affinity of the antibody–antigen interaction in our system is lower than that of the hen egg lysozyme system (D.N., unpublished results) and so suggest that it is the form of the autoantigen, rather than its affinity for the B-cell receptor, that is important for deletion. Preliminary results with our anti-H-$2K^k$ transgenic mice crossed to a Tg mouse producing 1–10 nM soluble H-$2K^k$ resulted in neither anergy nor clonal deletion (D.N., B. Arnold, and G. Hammerling, unpublished data).

These results strongly support the suggestion (2, 16–20) that deletional tolerance occurs in the pre-B-cell to B-cell transition. Future experiments using this system will study the fate of B cells that encounter autoantigen at a later developmental stage.

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