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Immunology

Immune deficiency due to high copy numbers of an A^k_β transgene

(class II/transgenic mice)

SUSAN GILFILLAN*, SADAKAZU AISO*, SARA A. MICHIE†, AND HUGH O. MCDEVITT*‡

Departments of *Microbiology and Immunology, ‡Medicine, and †Pathology, Stanford School of Medicine, Stanford, CA 94305

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ABSTRACT **Because allelic polymorphism of the major histocompatibility complex class II antigens affects the immune response at several levels, we wished to characterize the contribution of a particular α or β chain *in vivo* using transgenic mice. We have established and characterized 12 lines of H-$2^{s/s}$ mice carrying from 1 to 65 copies of an A^k_β transgene. The transgene was coexpressed with the endogenous allele in a tissue-specific manner, and A^k_β mRNA expression correlated well with transgene copy number. High copy number (extreme overexpression) of the transgene was associated with a variety of defects, including a significant reduction in Ia cell-surface expression, a severe decrease in B-cell number, abnormal extramedullary granulopoiesis, and an increased susceptibility to infection. In this paper we describe in detail the phenotype associated with high copy numbers of the A^k_β transgene. The defects we have observed may be relevant to similar phenomena seen in other transgenic mice. In addition, these mice have fortuitously provided a system in which to assess the effect of various levels of class II cell-surface expression in the thymus on selection of the T-cell repertoire.**

The major histocompatibility complex class II (Ia) antigens are highly polymorphic, and the alleles expressed within an individual animal largely determine its ability to respond to particular antigens (1). Two class II isotypes are expressed in the mouse, I-A and I-E. Each is a cell-surface glycoprotein composed of noncovalently associated α (33–35 kDa) and β (27–29 kDa) chains (2, 3). With the exception of E_α, all chains are polymorphic, and most of the allelic sequence differences are clustered in the membrane-distal domain of each chain (α1 and β1). A structural model for the class II antigens (4) has been derived from the class I HLA-A2 crystal structure (5), which suggests that the α1 and β1 domains interact to form a peptide-binding groove on the outer surface of the molecule. Functionally, these domains are important for T-cell recognition and α/β chain pairing (6–8). In addition, the level of any particular class II heterodimer expressed on the surface of antigen-presenting cells is critical in that a minimum threshold is required for T-cell activation (9–12).

Because the class II antigens affect the immune response at several levels, the contributions of specific amino acids or epitopes to peptide binding, T-cell recognition, and α/β chain pairing have been the subject of intense research. To extend these studies to an *in vivo* system, we introduced an A^k_β genomic clone into the germ line of H-$2^{s/s}$ mice (which do not express I-E) and have established 12 independent lines carrying from 1 to ≈65 copies of the A^k_β transgene. The transgene was coexpressed with the endogenous allele, and A^k_β mRNA expression correlated well with transgene copy number. High copy number (extreme overexpression) of the transgene was deleterious in these mice and interfered with cell-surface expression of I-A and with B-cell differentiation at two distinct stages. This B-cell depletion was accompanied by a variety of defects, including increased numbers of granulocytes, abnormal extramedullary hematopoiesis (primarily granulopoiesis), and an increased susceptibility to infection.

The defects we have observed can clearly be attributed to overexpression of the A^k_β transgene, and the phenotype described may be relevant to similar phenomena observed in other transgenic mice. The effect of increasing levels of A^k_β mRNA expression on α/β chain pairing and cell-surface expression will be described in detail elsewhere (13). Because cell-surface levels of Ia can be modulated by breeding appropriate pairs, these mice also provide a well-controlled system in which to assess the effect of extreme variation in Ia cell-surface expression in the thymus on the selection of the T-cell repertoire.

MATERIALS AND METHODS

Mice. SJL, BALB/c, and 129 strain mice were obtained from The Jackson Laboratories, and B10.S mice from Olac (Bicester, U.K.). B10.A(4R) and F_1 offspring from these strains were bred and maintained in our colony. A^k_α transgenic mice (from the A^k_α-26 line; ref. 14) were provided by D. Mathis and C. Benoist.

Production of Transgenic Mice. An 11.6-kilobase (kb) fragment of the pI-A^k_β-1 genomic clone (15) containing 2–3 kb of 5′ sequence and 1–2 kb of 3′ sequence was purified and microinjected into the pronuclei of (B10.S × SJL)F_2 embryos using standard techniques (16, 17). Founders carrying the transgene were identified by blotting (18) *Bgl* II-digested genomic DNA extracted from tail tissue. Filters were hybridized with a full-length ^{32}P-labeled A^k_β cDNA clone. Copy number was determined in representatives from each line of mice (13).

Monoclonal Antibodies (mAbs). mAb 10–3.6 (anti-$A^{s,k,f,r,u}_\beta$, ref. 19), mAb 40.N (anti-$A^{k,f,r,u}_\beta$, ref. 20), and mAb MKS4 (anti-$A^{s,f,u}_\beta$, ref. 21) were grown in ascites and purified by ammonium sulfate precipitation followed by protein A affinity chromatography. Preparations were directly biotinylated (22). mAb RA3-6b2 (anti-B220, ref. 23), mAb MI/70 (anti-Mac 1, ref. 24), anti-CD5 (Ly1, ref. 25), mAb AF6-122 (anti-IgD, 5b; ref. 26), and mAb 33.12 (anti-IgM, ref. 27) antibodies were provided by A. Stall and N. Uchida (Stanford University). Anti-CD4 (L3T4) and anti-CD8 (Ly2) were purchased from Becton Dickinson.

Fluorescence-Activated Cell Sorter (FACS) Analysis. FACS analysis was done as described by Hayakawa *et al.* (28). Cells were analyzed on a dual-laser FACS IV (Becton Dickinson Immunocytometry) equipped with a logarithmic amplifier. Dead cells were excluded by propidium iodide staining.

Immunohistochemistry. Tissue samples were cut into small pieces, embedded in OCT-compound (Tissue-Tek; Miles Scientific), and frozen in dry ice/isopentane without fixation.

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Abbreviations: NT, nontransgenic; 4R×NT, [B10.A(4R) × NT]F_1; FACS, fluorescence-activated cell sorter; SPF, specific pathogen free; mAb, monoclonal antibody; (H,G)-A--L, (histidine, glutamic acid)-alanine--lysine.

Cryosections 4 μm thick were cut and air dried for 1 hr, fixed with cold acetone for 10 min, and washed three times in phosphate-buffered saline (PBS). For staining, the sections were first blocked with 1% bovine serum albumin (fraction V; Sigma) and then incubated for 30 min in an appropriate dilution of a biotinylated antibody. Slides were washed with PBS, then incubated with avidin–peroxidase for 30 min, washed, and developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma). Slides were then rinsed in H_2O and mounted under coverslips.

RESULTS

Transgene Expression. A^k_β mRNA expression correlated well with transgene copy number. In mice carrying one copy of the transgene, the ratio of A^k_β to A^s_β mRNA was <1 and progressively increased in mice carrying more copies of the transgene. The ratio of A_β to A_α mRNA increased from 1 to >60 in mice carrying from 1 to 65 copies of the transgene (13).

Cell-surface expression of the transgene was dependent on copy number and mRNA expression (Fig. 1). Mice carrying 1 copy of A^k_β expressed low levels of the transgene and control levels of A^s_β in the thymic cortex and medulla. Approximately equal levels of the transgene and endogenous allele were observed in the cortex and medulla from mice carrying 10 copies of A^k_β, but the levels of both A^k_β and A^s_β were lower than those observed in [B10.A(4R) × nontransgenic (NT)]F_1 (4R×NT) controls. No further increase in thymic A^k_β cell-surface expression was observed in mice carrying >10 copies of the transgene. However, cell-surface levels of A^s_β (and total Ia) were significantly reduced in these mice. This reduction in total Ia expression was confirmed with mAb 10-3.6 (anti-$A^{k,s,f,r,u}_\beta$; data not shown) and was more marked in the thymic cortex. Mice carrying 60–130 copies of A^k_β had a severe reduction in cell-surface expression of Ia in the thymic cortex and slightly decreased expression in the medulla. Extremely high levels of A^k_β were observed in both the cortex and medulla of double-transgenic mice carrying 65 copies of A^k_β and 20 copies of A^k_α. Cell-surface expression of A^s_β was markedly reduced in these mice.

This pattern of Ia cell-surface expression was also observed on B cells (13). Together, the mRNA and cell-surface expression data indicated that A^s_α/A^k_β chain pairing was possible but inefficient in these mice. A significant fraction of those chains competing for pairing did not reach the cell surface, particularly in the high-copy-number animals. Improperly paired and dissociated chains could not be detected intracellularly and appeared to be degraded quite rapidly (13).

Characterization of the Lymphoid System. Lymphoid cells from members of each transgenic line were characterized using the mAbs described. Despite varying levels of Ia in the thymus, the numbers of $CD4^+$ and $CD8^+$ T cells were not significantly different from controls in any A^k_β transgenic mice examined (Table 1). Although the ratio of $CD4^+$ to $CD8^+$ T cells was slightly high in several A^k_α/A^k_β double-transgenic mice, analysis of thymocytes, lymph nodes, and peripheral blood from 15 additional high-copy-number A^k_β and A^k_α/A^k_β double-transgenic mice (which include both the high and low extremes of Ia expression in the thymus) revealed no consistent differences in these T-cell populations.

Unexpectedly, a significant decrease in B-cell number was observed in mice carrying from 20 to 65 copies of the transgene. Mice carrying 50–65 copies of A^k_β had <40% the number of B cells seen in controls (Table 1). In addition, pre-B cells (identified as $IgM^-B220^{dull}Ia^-$) represented a relatively high percentage of the $B220^+$ cells observed in the spleens of high-copy-number animals (data not shown). Analysis with anti-IgM and anti-IgD antibodies demonstrated that the IgM^+IgD^+ B-cell population was selectively depleted in mice carrying from 10 to 65 copies of the transgene. Numbers

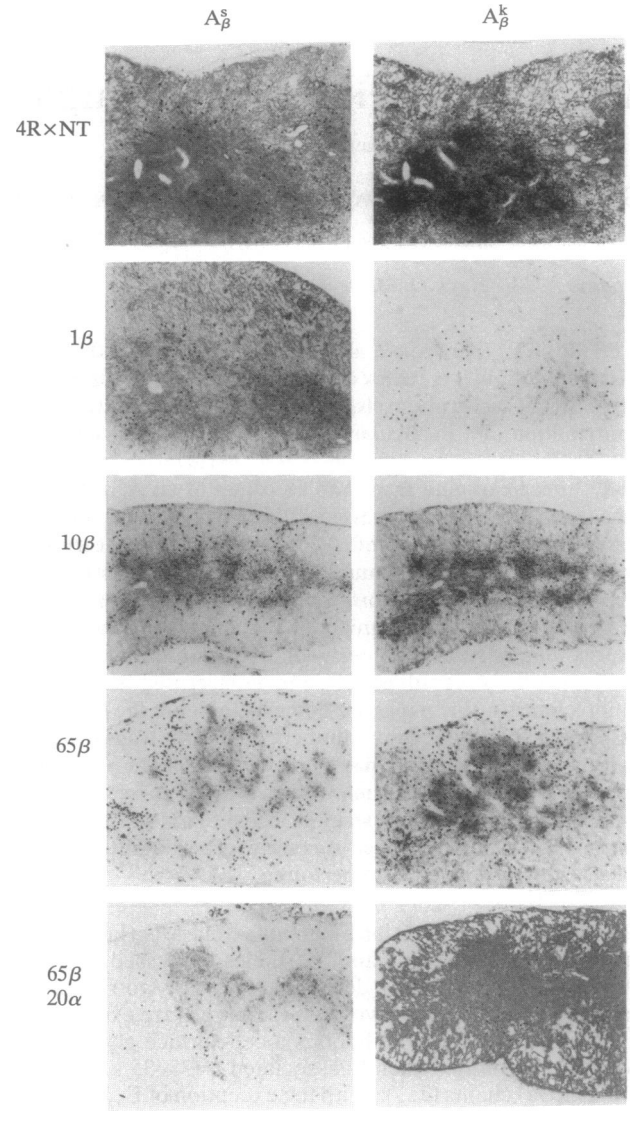

FIG. 1. Transgene and endogenous Ia expression, detected by immunoperoxidase staining, in thymus sections from A^k_β transgenic mice. Thymus sections from a 4R×NT control, A^k_β transgenic mice, and an A^k_α/A^k_β double-transgenic mouse were stained using mAbs MKS4 (A^s_β) and 40.N (A^k_β). Transgene copy numbers are indicated. The heavily stained single cells in all sections are granulocytes, which have endogenous peroxidase activity.

ranged from 70% to 10% of control values (Fig. 2). The IgM^+IgD^{dull} population was not depleted in any of the A^k_β mice and represented a relatively high percentage of B cells in mice carrying from 20–30 copies of the transgene. IgM^+IgD^{dull} cells comprised 17% rather than 6–7% of the total spleen-cell population in these mice. The decrease in B-cell number was accompanied by a significant increase in Mac 1^+ cells, the majority of which were identified as granulocytes in histological sections (Fig. 3). In addition, "null" cells (not stained by any of the antibodies used) comprised ≈10% of the spleen-cell population in control mice and 20–25% of spleen cells in the high-copy-number A^k_β mice.

To determine at which stage B cells were being depleted in the high-copy-number animals, bone marrow from representatives of each line was analyzed. No significant differences in pre-B cell numbers (IgM^-B220^{dull}) were observed in any of the transgenic mice. However, the numbers of immature B cells (IgM^+B220^+) were significantly reduced in mice carrying from 50–65 copies of the transgene (Fig. 4). The ≈60% reduction of this population in the bone marrow correlated

Table 1. FACS analysis of spleen cells from control and transgenic mice

Mice	Copy number, A^k_α/A^k_β	B220$^+$, %	Mac 1$^+$, %	CD4$^+$, %	CD8$^+$, %
		Control			
4R×NT, B10.A(4R)	1/1, 2/2	39 ± 7 (15)	9 ± 2 (10)	27 ± 7 (7)	18 ± 3 (7)
NT	0/0	40 ± 7 (16)	9 ± 3 (12)	28 ± 5 (12)	14 ± 4 (12)
		Transgenic			
High copy					
124, 130, 110, 111, 115	0/1–6	35 ± 5 (13)	10 ± 4 (6)	32 ± 6 (12)	12 ± 3 (12)
145	0/10	31 ± 5 (5)	12 ± 6 (4)	34 ± 3 (4)	13 ± 1 (4)
99, 92	0/20–30	**28 ± 3** (9)	**14 ± 3** (6)	26 ± 6 (7)	16 ± 5 (7)
34, 40, 105	0/50–65	**15 ± 6** (13)	**26 ± 7** (10)	26 ± 4 (5)	15 ± 2 (5)
26	20/0	28 ± 5 (4)	11 ± 1 (4)	37 (2)	17 (2)
105/40 × 26	20/65	**18 ± 4** (5)	**18 ± 5** (5)	36 (1)	13 (1)

Numbers that differ significantly from control values ($P < 0.01$, two-sided Student's t test) are in boldface type. The number of animals examined in each group is in parentheses beneath each average value. Similar numbers of cells were obtained from the spleens of all animals analyzed.

well with the decreased numbers of B cells observed in the periphery. Mice carrying from 20–30 copies of A^k_β had a similar, but less severe, phenotype. The depletion of B cells in the bone marrow coincided well with the onset of Ia expression, which occurs as pre-B cells begin to express cell-surface IgM (refs. 29 and 30; data not shown).

Additional Defects in High-Copy-Number A^k_β Mice. A variety of defects were observed in addition to (or perhaps as a result of) B-cell depletion in the high-copy-number mice. Initial breeding, particularly attempts to establish homozygous lines, indicated that the premature death rate among mice carrying high copy numbers of the transgene was extremely high (31). Because crosses between, as well as within, lines produced the same effect, integration site(s) did not appear responsible for this phenomenon. Six of the dead mice were autopsied at 3–4 months of age; all had extramedullary granulopoiesis in the liver, five had congestion and atelectasis in the lung, and three had extramedullary hematopoiesis and granulatomous inflammation within the myocardium. Other findings were consistent with viral or bloodborne bacterial infections. Mice carrying >150 copies of the transgene became ill and generally died at 3–4 weeks of age. Three that were autopsied had a severe myeloproliferative disease involving granulocytes.

Because the autopsy reports implicated a variety of infectious agents and mice carrying >100 copies of the transgene were difficult to maintain in our conventional facility, a line carrying ≈65 copies of A^k_β was established and bred in specific pathogen-free (SPF) conditions. Survival improved considerably, suggesting that an increased susceptibility to infection is one result of the defects seen in these mice. Though several homozygotes were identified and bred, they were difficult to maintain even in SPF conditions. One mouse autopsied after dying in the SPF facility shared many clinical findings with those dying in the conventional facility. Thus,

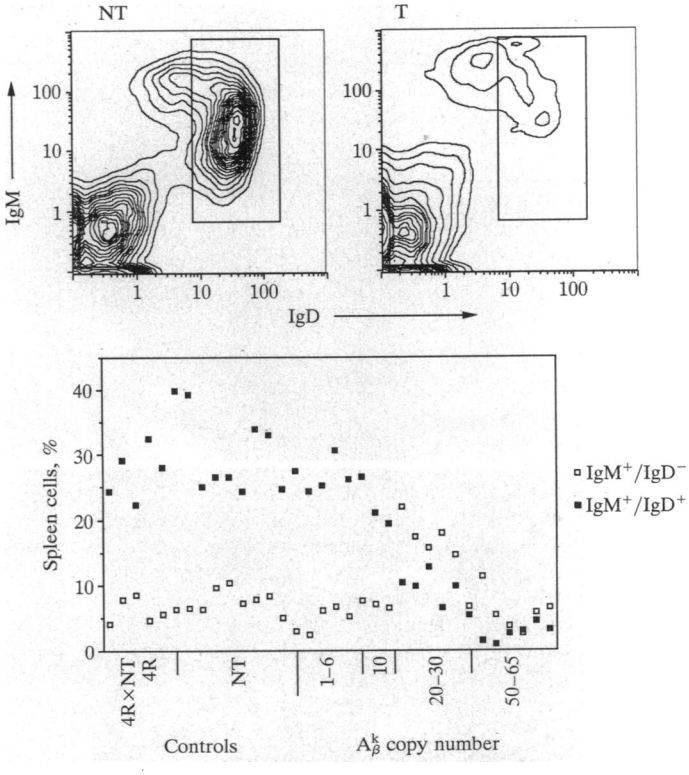

FIG. 2. Depletion of IgD$^+$ B cells in A^k_β transgenic mice. (*Upper*) Representative FACS plots of spleen cells stained with anti-IgM and anti-IgD from a NT control mouse and transgenic littermate (T) carrying 65 copies of the A^k_β transgene. Thirty thousand cells per sample were analyzed, and the data are displayed in 5% probability contour plots with the logarithm of fluorescence intensity on each axis. Boxes delineate the IgM$^+$/IgD$^+$ B-cell population in these samples. (*Lower*) Compiled data from representatives from other lines of A^k_β mice analyzed in the same manner are displayed in the scatter plot. 4R, B10.A(4R).

mice carrying ≈130 copies of the transgene may be susceptible to normally nonpathogenic bacteria present in the SPF colony or have more severe intrinsic defects caused by the transgene.

DISCUSSION

Correlation of mRNA expression with copy number has been reported for several other transgenes (32, 33), and DNase-1 hypersensitive sites conferring this property have been identified in flanking regions of the human β globin and *CD2* genes (34, 35). Although several transgenes have caused severe perturbation of the immune system, no defect has been associated with tissue-specific overexpression of the transgene. Depletion of the conventional B-cell population has been seen in mice expressing relatively high levels of a functionally rearranged IgM heavy-chain transgene (36). However, the severity of B-cell depletion did not appear to correlate with transgene expression levels in these mice (36, 37).

In our study, mice carrying >50 copies of the A^k_β transgene shared several defects, including variable but significant reductions in B-cell number, increased numbers of granulocytes, and abnormal extramedullary hematopoiesis (primarily granulopoiesis) in spleen, lymph nodes, and liver. Analysis of bone-marrow chimeras in which nontransgenic littermates were reconstituted with bone marrow from high-copy-number mice has indicated that these defects can be transferred with bone-marrow cells. This phenomenon does not appear to be an artifact unique to this particular trans-

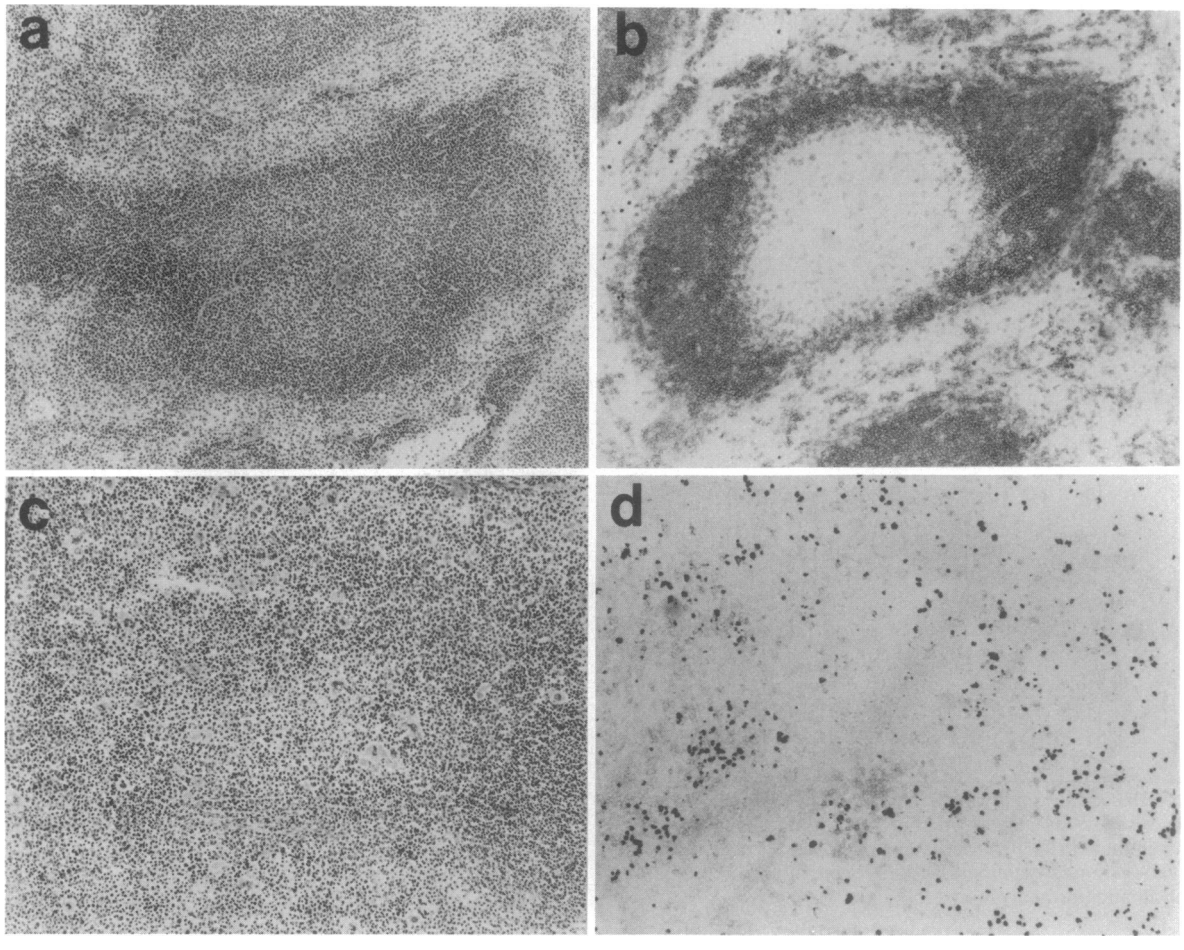

FIG. 3. B-cell depletion in a high-copy-number A^k_β transgenic mouse. Spleen sections from a 4R×NT mouse (*a* and *b*) and an A^k_β-40 mouse carrying 60–65 copies of A^k_β (*c* and *d*) were stained with hematoxylin/eosin (*a* and *c*) or mAb RA3-6b2 (anti-B220) (*b* and *d*). The heavily stained single cells in all sections are granulocytes, which have endogenous peroxidase activity.

gene, as we have produced a similar phenotype by introducing an A^d_β gene into NOD mice (H. Acha-Orbea and H.M., unpublished data). In addition, the A^k_β transgene-encoded protein appeared structurally normal, and immunization experiments using the I-A^k-restricted synthetic polymer (histidine, glutamic acid)-alanine--lysine [(H,G)-A--L] demonstrate that it could contribute to a functional A^k_α/A^k_β heterodimer in double-transgenic mice (31).

The A^k_β transgene appeared to be expressed in appropriate tissues but at extremely high levels. Modulating cell-surface expression of the transgene by crossing A^k_β mice to A^k_α or B10.A(4R) mice did not abrogate the defects observed in the high-copy-number mice. Interestingly, the defects were slightly less severe in the high-copy-number double-transgenic mice. Therefore, the extent of B-cell depletion and related defects observed in these mice correlates with the amount of unpaired A^k_β chains rather than overexpression of total class II protein. This suggests that overexpression of unpaired β chain functionally impairs at least some of those cells in which it is expressed.

B cells were affected at two distinct stages of maturation. The differentiation of IgD-expressing B cells was inhibited in mice carrying >10 copies of the transgene, and a progressive reduction in IgD^+ cell number correlated well with transgene copy number (and mRNA expression). The pre-B cell (IgM^-B220^{dull}) population in the bone marrow was not affected in any line of mice, but a significant decrease in immature B cells (IgM^+B220^+) was observed in mice carrying >50 copies of the transgene. The depletion observed at this stage correlated well with the onset of Ia expression in B cells (29, 30). Those B cells remaining in the periphery of the high-copy-number mice appeared functional. Secondary antibody responses to bovine serum albumin were elicited in high-copy-number A^k_β transgenic mice. Similarly, double-transgenic mice carrying 65 copies of A^k_β and 20 copies of A^k_α responded to (H,G)-A--L. Anti-(H,G)-A--L antibody titers were lower than those of controls in several of the high-copy-number double-transgenic mice, probably reflecting more severely depleted B-cell numbers in those individuals (31).

Though the mechanism for B-cell depletion in the A^k_β mice is not clear, this phenomenon is reminiscent of that observed in transgenic mice in which insulin-secreting beta cells are functionally impaired and/or depleted by insulin promoter-driven expression of major histocompatibility complex class II and class I proteins (38–41). Thus, high-level expression of class I or class II proteins can functionally impair and/or kill pancreatic beta cells, presumably by disrupting essential intracellular processes (for discussion, see ref. 41). Whereas any damage to beta cells that affects insulin secretion will result in characteristic "diabetes," similar impairment of Ia-expressing cells would be phenotypically more complex. The B-cell depletion and other defects we have observed in the high-copy-number A^k_β transgenic mice are consistent with such a phenomenon.

Whether other Ia-expressing cells in the high-copy-number A^k_β mice are functionally intact is not known. Thymus tissue from several of these mice appeared normal (except for class II expression) in histological sections. Macrophage numbers were not reduced. The increase in granulocyte number may be a secondary defect. Depletion of B cells in these animals

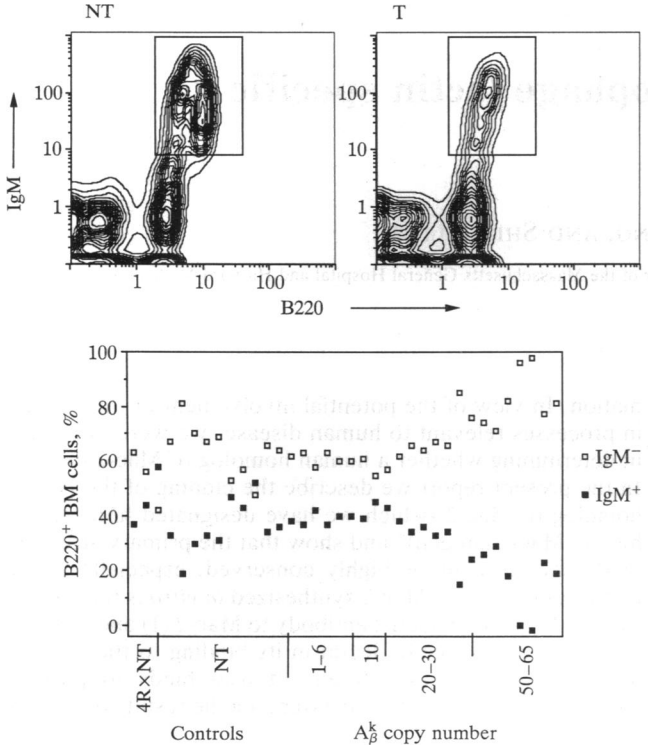

FIG. 4. Depletion of IgM^+ $B220^+$ cells in the bone marrow of high-copy-number A^k_β mice. (*Upper*) Representative FACS plots of bone-marrow (BM) cells stained with anti-IgM and anti-B220 from a NT control and transgenic littermate (T) carrying 65 copies of the A^k_β transgene. To emphasize the $B220^+$ cell population, only cells with low forward and wide-angle light scatter are displayed (36). Thirty thousand bone-marrow cells were analyzed for each sample, and data are displayed in 5% probability contour plots with the logarithm of fluorescence intensity on each axis. The boxes delineate the IgM^+ population. (*Lower*) Representatives from each copy-number range were analyzed in the same manner, and compiled data are displayed in the scatter plot; percentages of IgM^+ vs. IgM^- $B220^+$ cells are plotted for each individual. $B220^+$ cells comprised 20–25% of the bone-marrow population in these mice.

may lead to the extramedullary hematopoiesis/granulopoiesis observed.

Collectively, these A^k_β mice illustrate some of the critical parameters and inherent limitations of approaching class II structure/function studies in transgenic mice. Copy number dictates the level of transgene expression and has a profound effect on phenotype in these mice. By crossing various copy numbers of the A^k_β transgene onto other haplotypes, pairing, expression, and the effect of various A_α/A^k_β heterodimers on T-cell selection and the response to defined antigens can be examined. In addition, these mice provide a well-controlled system in which to assess the effect of extreme variation in Ia cell-surface expression in the thymus on selection of the T-cell repertoire.

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