Rearrangement and expression of endogenous immunoglobulin genes occur in many murine B cells expressing transgenic membrane IgM

(allelic exclusion/transgenic mice/fluorescence-activated cell sorting)

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ABSTRACT   Transgenic mice carrying immunoglobulin genes coding for μ heavy chain and κ light chain have been used to study the mechanisms involved in allelic and isotypic exclusion. We report here that individual cells from transgenic mice carrying a functionally rearranged μ heavy chain gene (capable of generating both membrane and secreted forms of IgM) can rearrange an endogenous μ heavy chain gene and simultaneously produce both transgenic and endogenous IgM. These "double-producing" cells express both endogenous and transgenic IgM in the cytoplasm (detected by immunohistology) and on the cell surface (detected by multiparameter fluorescence-activated cell sorter analysis). In addition, they secrete mixed IgM molecules containing both transgenic and endogenous μ heavy chains (detected in serum by radioimmune assay). The transgenic mice studied also have relatively large numbers of cells that produce endogenous immunoglobulin in the absence of detectable transgenic immunoglobulin ("endogenous-only cells"). The mechanisms that generate double-producing cells and endogenous-only cells appear to be under genetic control because the frequencies of these B-cell populations are characteristic for a given transgenic line. Thus, our findings indicate that more is involved in triggering allelic exclusion than the simple presence or absence of membrane μ heavy chains (as has been previously postulated).

Murine B cells generate by rearrangement a functional immunoglobulin heavy chain gene (*Igh*) on only one of the *Igh*-homologous chromosomes (1, 2). This results in each B cell producing antibodies encoded by only one of two potential *Igh-6* alleles encoding IgM (3, 4), a phenomenon referred to as allelic (or haplotype) exclusion (4, 5). The nature of the signals and/or mechanisms involved in allelic exclusion is not well understood; however, one commonly accepted view holds that the presence of the product of a functionally rearranged *Igh* gene prevents further rearrangement of the *Igh*-chromosomal region (1, 6).

Recently, a series of studies has been published using transgenic mice carrying a functionally rearranged μ heavy chain gene to test this hypothesis (6–11). Data from these studies have generally appeared consistent with the overall hypothesis; however, it is difficult to draw a firm conclusion from the evidence presented. Three potential problems cloud interpretation of these data: (*i*) in most of the previous studies, the expression of transgenic and endogenous IgM in individual B cells was primarily determined by analysis of cell lines (Abelson transformed lines or hybridomas) derived from the transgenic mice and are not necessarily representative of the B-cell populations actually present in the

animals (7–11); (*ii*) several of the transgenic lines studied were made with outbred (F$_1$) founder mice (6, 8–10) and, thus, will have considerable genetic variability, which may affect B-cell development; and (*iii*) our recent studies (with the M54 and M95 inbred transgenic mouse strains) demonstrate that the introduction of the immunoglobulin transgene can selectively block development of B-cell subpopulations (12).

In studies presented here, we reopen the question of whether the presence of a functionally rearranged μ heavy chain transgene ("μ transgene") is sufficient to block endogenous *Igh* gene rearrangement during B-cell development. Using anti-IgM allotypic reagents in multiparameter fluorescence-activated cell sorting (FACS), RIA, and immunohistological analyses, we directly measure endogenous and transgenic IgM production in individual B cells in the M54 and M95 μ transgenic mouse lines. With the added resolution provided by these methods, we show that the presence of the μ transgene does not always block endogenous gene rearrangement. That is, in addition to cells that exclusively produce the transgenic IgM, we find cells that produce both transgenic IgM and endogenous IgM or simply produce endogenous IgM without detectable levels of the transgene product. We discuss the significance of these three types of cells in the μ transgenic mice with respect to current theories of allelic exclusion.

MATERIALS AND METHODS

Animals. BALB/c, (BALB/c × C.B-17)F$_1$ and (SJL × BALB)F$_1$ were bred and maintained in the Herzenberg facility. BALB/c mice are *Igh-C^a* immunoglobulin haplotype (C = constant region), while SJL and C.B-17 are *Igh-C^b* haplotype (13). M54, M95, M94, and M52 transgenic lines were provided by R. Grosschedl (University of California, San Francisco). The development of these lines has been described in detail (14). The transgenic μ234-4 mice were generously provided by U. Storb (University of Chicago) and R. Brinster (University of Pennsylvania) (9).

Antibodies. The monoclonal mouse antibody AF6-78.25 is specific for IgM of *Igh-C^b* haplotype strains of mice (15). The mouse monoclonal antibody DS-1 is specific for IgM of *Igh-C^a* haplotype mice (D.G.S., unpublished data). Both antibodies are IgG1 isotype. Purification and fluorochrome conjugation of the monoclonal antibodies have been described in detail (16).

FACS Analysis. Single-cell suspensions were prepared from lymphoid organs as described (17) and stained with

Abbreviations: FACS, fluorescence-activated cell sorting; μ transgene, μ heavy chain transgene; C, constant region.
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optimal amounts of the anti-Igh-6a fluorescein isothiocya-nate-conjugated DS-1 antibody and the anti-Igh-6b biotin-conjugated AF6-78.25 antibody. Biotin-conjugated anti-Igh-6b was revealed with Texas red-avidin. FACS analyses were conducted as described (17, 18). Dead cells were stained with propidium iodide and excluded from the analyses (18). For each analysis, data from 30,000 viable cells were collected. Data are presented as 5% probability contour maps (19).

Immunohistology. Single-cell suspensions were prepared from spleen. Cell surface immunoglobulin was capped by incubating an aliquot of the cell suspensions with monoclonal rat anti-mouse IgM at 37°C for 60 min. Cytocentrifuge preparations were made by spinning down $\approx 10^6$ cells onto microscope slides. For double immunofluorescence, ace-tone-fixed (10 min at room temperature) cytospots were incubated for 30 min at room temperature with fluorescein-anti-Igh-6a and biotin-anti-Igh-6b and then washed. The biotin-anti-Igh-6b was revealed with Texas red–avidin. After extensive washing with phosphate-buffered saline (PBS; overnight at 4°C), cytospots were mounted in 90% (vol/vol) glycerol in PBS. Proportions of cells positive for cytoplasmic staining of endogenous IgM (Igh-6b, red fluorescence) and/or transgenic IgM (Igh-6a, green fluorescence) were determined by fluorescence microscopy. For each sample, at least 250 cytoplasmic IgM-positive cells with plasma cell-like morphology (large amounts of cytoplasm, eccentric nucleus) were analyzed.

Radioiodination of Antibodies. Purified AF6-78.25 and DS-1 were labeled with ^{125}I by the method of Hunter and Greenwood (20).

Solid-Phase RIA. Polystyrene microtiter plates were coated with either AF6-78.25 or DS-1 as described (21). After incubation to allow binding of serum IgM and washing,

radiolabeled AF6-78.25 or DS-1 was used to detect bound IgM. As an isotype control for nonspecific binding to AF6-78.25 or DS-1, which are both IgG1 antibodies, parallel assays were performed using MOPC-21 (an IgG1 myeloma protein) as the plate coat. Nonspecific binding (typically <100 cpm) was subtracted from the test values.

RESULTS

Transgenic and Endogenous Cell-Surface IgM Are Frequently Coexpressed on B Cells. The transgenic mouse lines M54 and M95 were generated by introducing a functionally rearranged BALB/c μ heavy chain gene into the germ line of a C57BL/6 inbred mouse. BALB/c mice express the *a* allele of the gene for IgM (*Igh-6a*), while C57BL/6 mice express the *b* allele (*Igh-6b*) (13). Thus, transgenic (Igh-6a) and endogenous (Igh-6b) IgM can be distinguished by using monoclonal antibodies specific for Igh-6a and Igh-6b (DS-1 and AF6-78, respectively).

Cells producing both transgenic and endogenous surface IgM are readily detectable by FACS analysis of the M95 and M54 μ transgenic mice. FACS contour plots of lymphoid tissues simultaneously stained for endogenous (Igh-6b) and transgenic (Igh-6a) IgM are shown in Fig. 1. Mice of both transgenic lines have three distinct populations of B cells: those expressing only transgenic IgM (Igh-6a only); those expressing only endogenous IgM (Igh-6b only); and double-producing cells that express both endogenous and transgenic IgM (Igh-6a/6b). In addition, all three populations were detectable by FACS analysis (data not shown) in the μ243-4 transgenic line developed by Brinster, Storb, and coworkers (9), which has a different μ transgene construct than the one in M54 and M95.
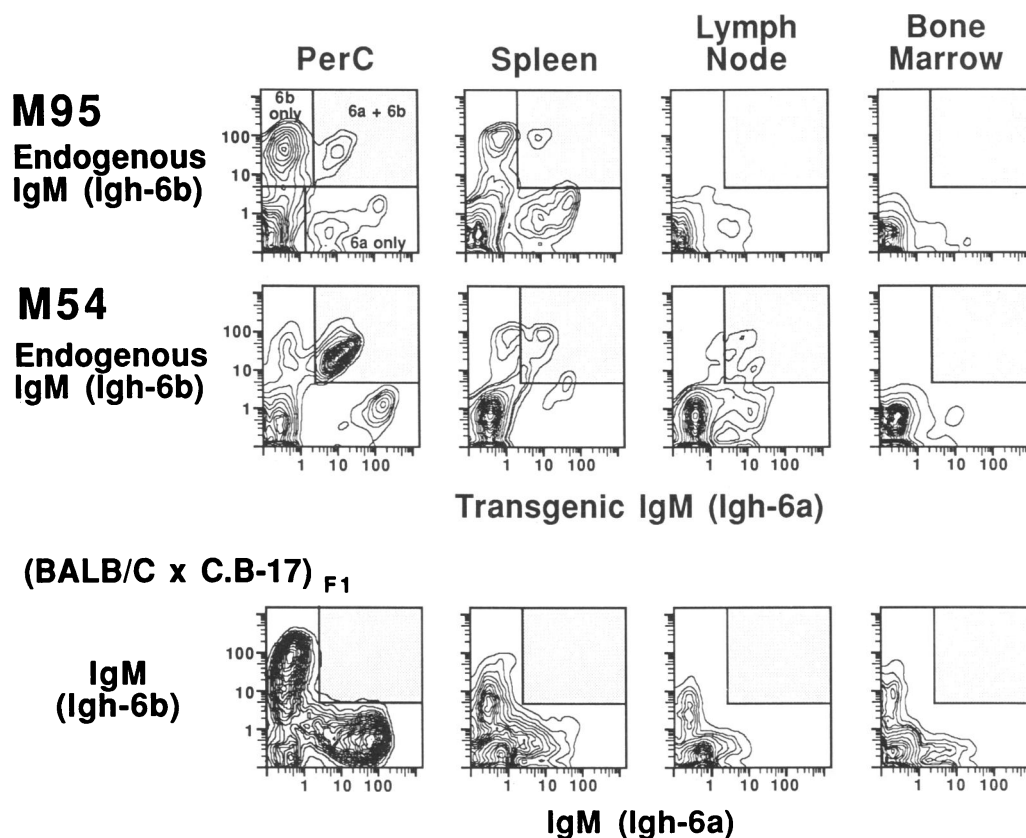

FIG. 1. FACS analysis of transgenic (Igh-6a) and endogenous (Igh-6b) IgM production in lymphoid tissues of transgenic and allotype-heterozygous mice. Gates used to determine the frequency of the Igh-6b-only, Igh-6a-only, and Igh-6a/6b cells are shown in row M95, panel PerC (peritoneal cells). The gray areas in each contour plot show the Igh-6a/6b region.

In contrast, FACS analyses of normal allotype heterozygotes, (BALB/c × SJL)F$_1$ or (CB.17 × BALB/c)F$_1$, revealed only the expected allelically excluded Igh-6a-only or Igh-6b-only B cells. Only background levels of cells that stain for both Igh-6a and -6b were detectable (<2% of B cells in peritoneum and <1% of B cells in spleen) (Fig. 1). Thus, the double-producing cells (15–80% of B cells in spleen and peritoneum) are unique in transgenic mice and are not present in animals in which only the normal mechanism(s) of allelic exclusion are functioning.

Transgenic and Endogenous IgM Are Coexpressed in Plasma Cell Cytoplasm. Simultaneous expression of transgenic and endogenous IgM was clearly detectable in the cytoplasm of plasma cells in transgenic mice (Fig. 2 and Table 1). In these studies, single-cell suspensions were prepared from the spleens of transgenic (M54, M95) and (BALB/c × SJL)F$_1$ control allotype-heterozygous mice, fixed to glass slides, and simultaneously stained for Igh-6a and -6b. Plasma cells showing a distinct cytoplasm with clear immunoglobulin staining were scored as Igh-6a only, Igh-6b only, or Igh-6a/6b. In M54 and M95 spleen, roughly 10% of the plasma cells scored fell into the double-producer category. No double producers were found in spleens from allotype-heterozygous controls (see Table 1). These findings confirm the presence of cells that simultaneously express endogenous and transgenic IgM in the transgenic mice and show that these cells can develop into plasma cells that presumably secrete immunoglobulin. In addition, these findings show that Igh-6a/6b doubly stained cells identified by FACS analysis are not the result of serum Igh-6a or -6b being passively (or actively) bound to the cell surface.

The relative frequencies of Igh-6a-only, Igh-6b-only, or Igh-6a/6b cells detected by this method underscore the problems inherent in using hybridoma cell lines produced from transgenic mice to assess the relative expression of endogenous and transgene *Igh-6*. In previous studies (11), no double-producing hybridomas were obtained from the M54 and M95 lines, even though these cells represent 15–40% of total B cells (Fig. 3) and roughly 10% of the plasma cells (Table 2) in these strains. Furthermore, 85% of the hybridomas obtained from M95 and M54 spleen and lymph node expressed endogenous μ heavy chain in the absence of the transgene [i.e., are Igh-6b only (11)], while only 15–40% of M95 and M54 spleen and lymph node B cells were Igh-6b only by FACS analysis (see Fig. 3). Thus, the hybridomas obtained were not representative of the distribution of endog-

Table 1. Transgenic and endogenous IgM coproduced in the cytoplasm of plasma cells

Strain	Cytoplasmic staining,* %		
	Igh-6a only	Igh-6a/6b	Igh-6b only
M95	24	10	66
M54	14	7	79
(BALB/c × SJL)F$_1$	57	0	43

Values shown are the percentages of splenic plasma cells (with large amounts of cytoplasm and eccentric nuclei) from transgenic and allotype-heterozygous mice that showed cytoplasmic staining for Igh-6a only, Igh-6b only, or Igh-6a/6b (see Fig. 2).
*Percentages are based on 250 cells counted for each strain.

enous gene- and transgene-expressing splenic and lymph node B cells (as defined by FACS analysis). This bias may be explained in part by the fact that Igh-6b-only cells constitute a substantially higher percentage of the cytoplasmic Ig$^+$ (activated) B cells than of the total surface Ig$^+$ B cells and, thus, may preferentially yield hybridomas.

Relative Frequency of B-Cell Types in Transgenic Lines. While all three types of B cells (Igh-6a only, Igh-6b only, and Igh-6a/6b) were present in both the M54 and M95 mice, the relative frequencies of the three types differed dramatically in each of the transgenic lines studied. For example, the peritoneal B cells of M95 mice were predominantly Igh-6b only (80%), while in M54 mice peritoneal B cells were almost exclusively Igh-6a/6b (85%) (Figs. 1 and 3). Similar differences were seen in the other lymphoid organs.

There was no apparent correlation of the frequency of Igh-6b-only and/or Igh-6a/6b cells in a given transgenic line with the number of copies of the transgene or the amount of transgene protein produced. The M52 and M95 lines, both of which had very high frequencies of Igh-6b-only cells, had 140 and 17 copies, respectively, of the transgene (14), while M54, which had predominantly Igh-6a/6b cells, had 30 copies. All three lines produced similar levels of serum transgenic IgM (ref. 14; Table 2).

The relative frequencies of Igh-6a-only, Igh-6b-only, and Igh-6a/6b B cells were reproducible and characteristic for each transgenic line. That is, M95 mice always showed high levels of Igh-6b-only cells, while M54 mice had high levels of double producers. The relative frequencies of each of the three cell types in another transgenic line, M52, were intermediate between those in M54 and M95 (data not shown). Thus, each transgenic line, even though produced with the
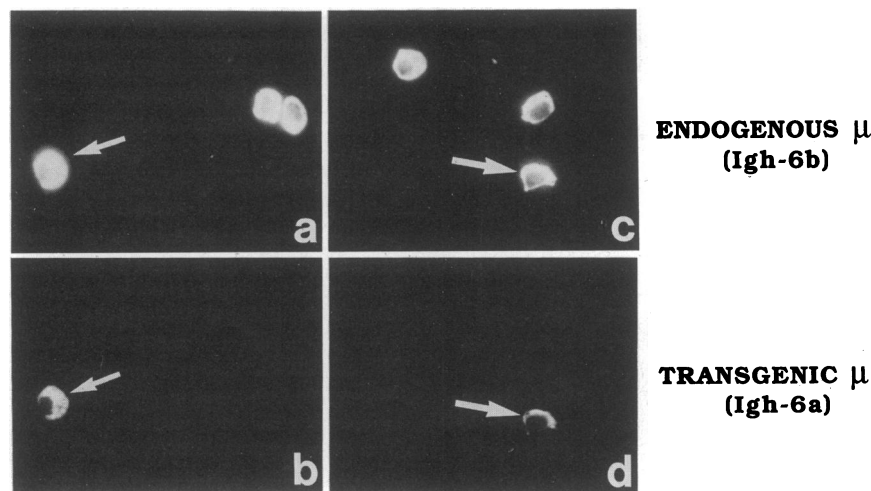

ENDOGENOUS μ
(Igh-6b)

TRANSGENIC μ
(Igh-6a)

FIG. 2. Simultaneous production of transgenic and endogenous IgM in the cytoplasm of splenic B cells from M95 transgenic mice, as demonstrated by double immunofluorescence staining. Fixed cytocentrifuge preparations (*a, b* and *c, d*) were simultaneously stained for Igh-6a (green fluorescence, *b* and *d*) and Igh-6b (red fluorescence, *a* and *c*). Each set of pictures shows both double-producer plasma cells (indicated by arrows) and cells containing only endogenous IgM.

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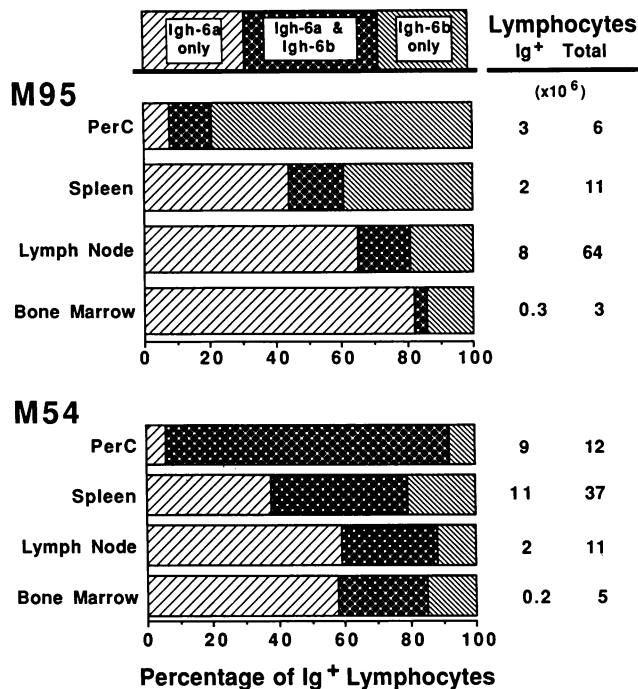*Proc. Natl. Acad. Sci. USA 85 (1988)* 3549

FIG. 3. Relative frequency of Igh-6a-only, Igh-6b-only, and Igh-6a/6b B cells in the lymphoid tissues of transgenic mice. Each bar graph shows the percentages of Ig$^+$ B cells in the designated tissue that produce only transgenic IgM (Igh-6a only), only endogenous IgM (Igh-6b only) or both (Igh-6a/6b). The values are the average of three mice, determined from FACS analysis as shown in Fig. 1. The number of Ig$^+$ B cells and total lymphocytes obtained from each organ is given to the right of each bar graph.

same gene construct, was unique. Therefore, heritable differences among the lines play a role in determining the frequencies of endogenous-gene-only and double-producer cells. While this could simply be due to different transgene insertion sites in the lines, it does suggest that multiple factors are involved in determining whether an endogenous gene is rearranged and/or expressed.

Distribution of the B-Cell Types in Lymphoid Organs. In addition to the differences among individual transgenic lines, there were striking differences in the relative frequencies of the three B-cell populations (Igh-6a only, Igh-6b only, and Igh-6a/6b) among the lymphoid organs within a given transgenic line (see Fig. 3). In M95 peritoneum, 92% of the B cells expressed endogenous IgM either alone (79%) or with transgenic IgM (13%). Only 8% of the peritoneal B cells showed transgene-induced allelic exclusion in the sense that they expressed transgenic IgM in the absence of endogenous IgM. In contrast, 65% of the B cells in the lymph node showed this type of allelic exclusion—i.e., expressed only the transgene.

This hierarchy in the frequency of transgene-only cells (peritoneum < < spleen < lymph node < = bone marrow) was found in every μ transgenic line we have studied (M54, M95, and M92). This may be because the transgene-only cells are not represented equally in all B-cell lineages or subpopulations [since the frequency of Ly-1 lineage B cells in the various lymphoid organs follows the same hierarchy (22)].

Transgenic Mice Produce Mixed IgM Molecules. IgM molecules that contain both endogenous and transgenic μ heavy chains were detectable by RIA in the sera of transgenic mice (Table 2). These "mixed" IgM molecules were revealed in RIA assays in which anti-Igh-6a was used as the plate coat antibody and radiolabeled anti-Igh-6b was used as the revealing antibody (and vice versa). In these assays, radioactivity is bound to the plate only if the serum contains (pentameric) IgM molecules that have endogenous and transgenic μ heavy chains and, thus, can bind to both the anti-Igh-6a and anti-Igh-6b antibodies.

Although sera from all transgenic animals tested had substantial levels of mixed IgM molecules, no mixed molecules were detected in sera from allotype-heterozygous [(BALB/c × SJL)F$_1$] mice in which the allelically excluded B cells produce levels of serum Igh-6a and Igh-6b equal to the overall levels in the transgenic mice. Furthermore, centrifuging the transgenic serum samples at 130,000 × g for 30 min did not result in a decrease in radioactivity bound in the mixed RIA, indicating that the mixed molecules are not due to Igh-6a–Igh-6b complexes. Thus, the double-producing cells detected in the transgenic mice by FACS and immunohistological analyses can be stimulated to secrete IgM molecules that reflect the joint expression of the transgene and a rearranged endogenous μ heavy chain gene.

DISCUSSION

Recently, several laboratories have reported data from studies with transgenic mice that show that the introduction of a functionally rearranged μ heavy chain gene interferes with endogenous gene rearrangement (6–10). These findings, which have been interpreted as indicating that the presence of membrane IgM is sufficient to prevent endogenous *Igh* rearrangement (6), would appear to be at variance with evidence presented here, which shows clearly that endogenous μ heavy chain gene rearrangement can and does occur frequently in B cells expressing transgene-encoded membrane IgM. However, reevaluation of the earlier data in the context of our findings with the M54 and M95 transgenic lines suggests that this variance is mostly at the interpretive level.

In essence, the simplest interpretation of the data we have presented is that the expression of membrane μ heavy chain is not sufficient to prevent rearrangement of endogenous *Igh* genes in all B cells. As we have shown, endogenous μ heavy chain is frequently produced in B cells that also produce the

Table 2. Transgenic mice produce IgM molecules containing both transgenic and endogenous μ heavy chains

	Igh-6		Plate coat Ab/^{125}I-labeled Ab, cpm bound			
Serum source	Endogenous μ allotype	Transgene μ allotype	$\dfrac{\alpha 6a}{\alpha 6a^*}$	$\dfrac{\alpha 6b}{\alpha 6b^*}$	$\dfrac{\alpha 6a}{\alpha 6b^*}$	$\dfrac{\alpha 6b}{\alpha 6a^*}$
M54	b	a	5870	5160	5240	3510
M95	b	a	7420	5160	6030	4680
M94	b	a	6200	3940	4520	1790
M54 sib	b	No transgene	0	5340	169	0
BALB/c	a	No transgene	8030	0	17	229
(BALB/c × SJL)F$_1$	a/b	No transgene	8530	5900	106	129

Values are the average of three to six 6- to 10-month-old animals. Serum samples were diluted 1:1250. See the text for explanation. Ab, antibody; α, anti.

transgenic μ chain. These double producers coexpress endogenous and transgenic IgM, both in the cytoplasm and on the cell surface. Furthermore, they secrete "mixed" IgM molecules (with transgenic and endogenous μ heavy chains) that are readily detectable in serum.

Data from the earlier studies have also consistently demonstrated the presence of B cells producing endogenous immunoglobulin in transgenic mice. In the transgenic mice of Nussenzweig *et al.* (6) that produce human membrane μ heavy chain, 10–30% of splenic B cells (5–15% of splenocytes) express endogenous μ heavy chain. While double producers were not detected in these animals, this may be due to the fact that the frequency of these cells may be very low in a given transgenic line, especially in the spleen (e.g., see the data for the M95 spleen in Fig. 1 and Table 2). No data were presented for analyses of peritoneal cells, where (as we have shown) double-producing cells are most readily detected.

Iglesias *et al.* (10) also demonstrated endogenous immunoglobulin production in *Igh* transgenic mice, in this case in animals carrying a productively rearranged δ heavy chain transgene (*Igh-5*). These authors conclude that the *Igh-5* transgene markedly blocks endogenous *Igh-6* rearrangement; however, approximately a third of the splenic B cells in these mice express endogenous *Igh-6*, and a significant fraction of these coexpress endogenous *Igh-6* and the transgenic *Igh-5*. Hybridoma studies have also revealed the existence of cells expressing endogenous *Igh* genes in transgenic mice (8, 11). Thus, although less attention has been paid to cells producing endogenous immunoglobulin in earlier studies of transgenic mice, rearrangement of the endogenous chromosome has not been completely suppressed in any *Igh* transgenic line studied.

Bringing the endogenous immunoglobulin production in μ transgenic mice into focus introduces a different perspective on studies of the mechanisms involved in allelic exclusion. The cells that express endogenous μ heavy chain gene in the absence of detectable amounts of transgenic μ heavy chain are perhaps the most intriguing in this respect. How does the expression of the functionally rearranged μ transgene become blocked in these cells? It is unlikely that they have physically lost the transgene, since the frequency of endogenous-gene-only cells appears to be under strict genetic and developmental control (i.e., the frequencies are unique in each transgenic line and in each lymphoid tissue within a given line). Thus, the loss of transgene expression in these cells could reflect the existence of a second allelic exclusion mechanism that operates in these mice at the level of gene expression rather than rearrangement.

Finally, the frequent appearance of cells producing both transgenic and endogenous IgM in several transgenic lines shows that the production of membrane μ heavy chain does not always result in allelic exclusion. This finding constitutes the severest challenge to the idea that the production of membrane μ heavy chain is sufficient to block subsequent μ heavy chain gene rearrangements in developing B cells. In essence, barring *ad hoc* hypotheses that allow for endogenous immunoglobulin gene rearrangement before sufficient levels of transgene membrane μ heavy chain are expressed, the existence of double-producing cells suggests at a minimum that allelic exclusion is mediated by a more complex mechanism than previously postulated (6).

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