

# A high frequency of hybridomas from M54 $\mu$ heavy chain transgenic mice initially co-express transgenic and rearranged endogenous $\mu$ genes

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## Abstract

The M54 transgenic mouse line, which carries the 17.2.25 Ig  $\mu$  heavy chain gene, rearranges Ig heavy chains and expresses both transgenic and endogenous  $\mu$ . B cell lineage development is selectively impaired in these mice and cells that simultaneously express transgenic and endogenous  $\mu$  ('double-producers') are common amongst the B cells and plasma cells that do develop. Weaver, Imanishi-kari, Baltimore and colleagues failed to obtain double-producing hybridomas from M54 mice; however, molecular and serologic studies presented here show that such hybridomas are readily generated. These hybridomas are extremely unstable and rapidly yield variants producing either transgenic or endogenous  $\mu$ . Therefore the stable cloned lines we obtained, like Weaver *et al.*, were almost all single or non-producers. We also found that the  $V_H$  gene usage in our hybridomas was skewed towards the  $J_H$  proximal ( $V_H$ Q52,  $V_H$ 81X) families, supporting the idea that the expression of the M54 transgene alters the endogenous Ig repertoire.

## Introduction

Although all mammalian cells have a pair of homologous chromosomes that carry genes coding for Ig heavy (IgH) chains, B cell developmental mechanisms preclude the expression of both homologs in a single cell. This phenomenon, commonly called 'allelic exclusion' (1) and sometimes referred to as Ig haplotypic exclusion (2), was recognized in the early 1960s in studies using antibodies to allotypic markers on IgH chain constant regions encoded by alleles in the IgH chromosome region. In essence, it reflects the successful rearrangement of variable (VDJ) and constant region genes on one of the IgH chromosomes (3) and the failure to complete VDJ rearrangement on the other. The successful rearrangement (VDJC) creates a functional  $\mu$  heavy chain gene that is expressed (in conjunction with  $V_{pre-B}$  and  $\lambda 5$ ) on B cells early in development and contains a VDJ segment that will be used in IgM and in all other isotypes produced by the progeny of the rearranged B cell (4).

Several investigators have suggested that the expression of

the  $\mu$  heavy chain gene during B cell development is sufficient to block rearrangement and thus exclude expression of IgH genes on the other homolog (see, for example, 5). To test this hypothesis Grosschedl *et al.* (6) produced several transgenic mouse lines carrying the 17.2.25  $\mu$  heavy chain transgene coding for anti-NP antibodies and capable of producing both membrane bound and secretory IgM. Studies with these mice indicated that the  $\mu$  transgene decreased endogenous IgH rearrangement but did not completely suppress it (6,7). Thus these findings precluded a simple view of allelic exclusion.

In further work with these mice, Weaver *et al.* demonstrated that hybridomas generated from the M54 strain express either the transgene or Ig encoded by endogenous genes, but never both (8). They also showed that the M54 endogenous V gene repertoire (extrapolated from the V gene expression in the hybridomas) is skewed towards expression of the  $J_H$  proximal  $V_H$  genes (in particular  $V_H$ 81X).

These findings, coupled with evidence from the Baltimore laboratory suggesting that B cell development was disrupted in M54 mice, motivated our initial studies with the M54  $\mu$  transgenic line (9). We had shown previously that murine B cells were subdivided into two subsets/lineages: conventional B cells, which comprise the vast majority of B cells in spleen and lymph node; and B-1 cells (formerly called Ly-1 or CD5 B cells) (10,11), which are the predominant B cells in the peritoneal and pleural cavities. A series of studies from other laboratories demonstrated that the proximal  $V_H$  genes are over-represented in B-1 (Ly-1<sup>+</sup>) B cell lines and in hybridomas produced from sources rich in B-1 cells (12,13). The demonstration of similar  $V_H$  gene usage in the M54 hybridomas by Weaver *et al.* suggested to us that B-1 cells might be over-represented in M54 mice and led us to begin an investigation of B cell development and B cell lineage representation in these mice.

Our initial studies, conducted in collaboration with Baltimore and Grosschedl, demonstrated that the presence of the transgene causes a severe reduction in the numbers of B cells and pre-B cells belonging to the conventional lineage but has little or no effect on the development of B-1 cells (9). In further studies we showed that the B cells contained three distinct populations: those that expressed only Igh-6a allotype  $\mu$  chains encoded by the transgene; those that expressed only Igh-6b allotype  $\mu$  encoded by rearranged endogenous genes; and a sizable number of 'double-producer' B cells on which both the transgene encoded and endogenously encoded  $\mu$  genes were readily detectable. Finally we confirmed the dual expression of endogenous and transgenic  $\mu$ , both by histochemical staining studies that revealed (plasma) cells with the two  $\mu$  heavy chain allotypes in the cytoplasm and by radioimmunoassays that detected 'mixed' (hybrid) pentameric IgM molecules containing both transgenic and endogenous  $\mu$  chains in M54 sera (14).

Our finding of double-producers contrasted with the results of Weaver *et al.* (8) who did not find hybridomas producing both  $\mu$  allotypes. This apparent discrepancy seemed to support assertions of irregularities in the data of that paper. Imanishi-Kari and colleagues (15) explained the discrepancy by suggesting that our observation of double-producing B cells could be an artifact caused by cross-reactive specificities of the endogenous IgM antibodies.

In the studies presented here analysis of hybridomas generated from M54 mice definitively demonstrate both serologically and molecularly the existence of double-producer B cells in M54 mice. Roughly 30% of M54 hybridomas initially express both the transgene and an endogenous  $\mu$  (measured as mRNA or secreted IgM); however, we show that these double-producing hybridomas convert so rapidly to cells producing only a single IgM species (either transgenic or endogenous  $\mu$ ) that they overwhelmingly tend to be lost when hybridomas are expanded and subcloned in the usual procedures used to derive and maintain stable hybridoma clones.

Analysis of the  $V_H$  genes used in these hybridomas confirms the findings of Weaver *et al.* (8) that there is an over-representation of the  $V_H81X$  and  $V_HQ52$  families. In addition, the percentage of peritoneal B-1 cells with the characteristic B-1 specificity for phosphatidylcholine (PtC) is significantly elevated in these animals.

Finally we show that essentially all peritoneal B cells which have rearranged and express endogenous  $\mu$  have the B-1a or B-1b

phenotype. Together with our earlier studies which show that conventional B cells reconstituted from M54 bone marrow show little or no expression of endogenous IgM, these findings demonstrate that functional expression of endogenous IgM is restricted or at least highly biased towards cells of the B-1 lineage.

In our analysis of the M54 mice we have confirmed most of the findings originally reported by Weaver *et al.* (8). In particular that the introduction of a functionally rearranged  $\mu$  gene in M54 mice results in a profound alteration in the expression repertoire of the B cells. We differ from that paper in two respects, one of fact and one of interpretation. Factually, in contrast to their study in which no double producing hybridomas were observed we can readily generate and detect such hybridomas. However, our data indicate that the failure to find double-producing hybridomas in the Weaver *et al.* study is not surprising, due to the unusually extreme instability of Ig expression inherent in these hybridomas.

In interpreting their findings Weaver *et al.* suggest that the alteration in the expressed endogenous repertoire could be the result of idiotypic mimicry of the introduced transgene (8). While our data does not disprove that hypothesis, we would alternatively propose that the altered repertoire is the result of the fact that most if not all of the endogenous Ig is produced by cells of the B-1 lineage, which are known to have a repertoire which is skewed to the J proximal  $V_H$  gene families.

## Methods

### Animals

C57BL/6 and M54 transgenic mice were bred and maintained in our facility. The development of M54 transgenic mice has been described in detail (6).

### Antibodies

The following mAb were used in this study. AF6-78.25 (mouse anti-6b; recognizes IgM of the Igh-C<sup>b</sup> allotype) (16), DS-1 (mouse anti-6a; recognizes IgM of the Igh-C<sup>a</sup> allotype) (17), 331.12 (rat anti-IgM; recognizes IgM of both Igh-C<sup>a</sup> and Igh-C<sup>b</sup> allotypes) (18), M1/70 (rat anti-MAC-1) (19), 53-7 (rat anti-CD5) (20). Purification and fluorochrome conjugation of the mAb have been described in detail elsewhere (21).

### FACS analysis

Single-cell suspensions were prepared from lymphoid organs as described (14) and stained with optimal amounts of fluorochrome [fluorescein (F<sup>I</sup>), allophycocyanin (A<sup>PC</sup>)]-conjugated or biotin-conjugated antibodies. Biotin conjugated antibodies were revealed with Texas Red-avidin. FACS analyses were conducted using a dual laser FACStar<sup>Plus</sup> (Becton-Dickinson, San Jose, CA) as described (22). Dead cells were excluded from analyses by propidium iodide staining. Data were analyzed using FACS/DESK (Stanford University, Stanford, CA) and unless otherwise noted are presented as 5% probability contour plots (23).

### Hybridoma fusion

C57BL/6 and M54 mice were primed by intraperitoneal injection with 10  $\mu$ g of LPS 2 days prior to hybridoma fusion. Splenocytes were prepared and fused with SP2/0 mouse myeloma cells using polyethylene glycol 1500. Hybridoma cells were plated in 3 x 96-well plates and selected in HAT medium as described (24).

Clones were assayed via ELISA for the production of IgM, Igh-6a and Igh-6b and if positive subcloned by limiting dilution at 0.6 cells/ml.

#### ELISA

Production of IgM by hybridoma clones was assayed in 96-well microtiter plates using rat anti-mouse IgM heavy chain antibody 331 as coating antigen at 5  $\mu$ g/ml (50  $\mu$ l/well). After incubation with antigen and blocking with 3% BSA and 0.2% Tween-20 in PBS with 0.02% sodium azide, the ELISA plates were washed with 0.02% Tween-20 in PBS. A total of 50  $\mu$ l of hybridoma supernatant was added to each well. The production of IgM of Igh-6a or Igh-6b allotype was investigated by incubating duplicate wells with optimal amounts of either biotin-conjugated DS-1 (anti-6a) or biotin-conjugated AF6-78.25 (anti-6b) revealing antibodies, followed by streptavidin – alkaline phosphatase. The substrate used in this assay was 4-methylumbelliferyl phosphate (Sigma) and the amount of enzymatic reaction was quantified in a Fluoroskan (Titertek) using 355 nm excitation and 460 nm emission filters. Hybridoma clones that were double-positive by our screening assays were subjected to subsequent rounds of a mixed sandwich ELISA using anti-6a antibodies as the coating antigen and biotin – anti-6b antibodies as the revealing antibodies, or vice-versa. The conditions of the assay were identical as in our screening assays. Controls for the ELISA include BALB/c anti-dextran antibodies 19.1.2 (Igh-6a) and C57BL/6 anti-dextran antibody 2.31.1 (Igh-6b) kindly provided by Dr Elvin Kabat.

#### Northern blot analysis

RNA was isolated from M54 spleen and hybridoma clones by standard procedure (25). Equal amounts of RNA (10  $\mu$ g) were loaded into each lane of a 1% agarose – formaldehyde gel, electrophoresed and transferred onto nitrocellulose overnight in 20  $\times$  SSC. After pre-hybridization, the blot was hybridized overnight at 45°C with random-primed, [<sup>32</sup>P]dCTP-labeled M54 transgene specific 17.2.25 V<sub>H</sub> probe kindly provided by Dr R. Grosschedl (University of California, San Francisco, CA).

The blot was washed twice in 2  $\times$  SSC, 0.1% SDS at 65°C for 15 min each time and exposed overnight at –70°C. Expression of endogenous Ig was assayed by sequentially re-probing the blot with V<sub>H</sub>81X and V<sub>H</sub>Q52 gene specific probes (26) provided by Dr Wexler (Cornell Medical Center).

#### Southern blot analysis

DNA was isolated from M54 mouse tissues and hybridoma clones as described (26) and restriction digested with *Sac*I. Equal amounts of DNA (10  $\mu$ g) were loaded into each lane of a 0.8% agarose gel, electrophoresed and transferred onto nitrocellulose membrane in 10  $\times$  SSC. Prehybridization and hybridization conditions were identical to those in the Northern blot analysis. Endogenous Ig gene rearrangement was assayed by using a random-primed, [<sup>32</sup>P]dCTP-labeled J<sub>H</sub> probe (a 2 kb *Bam*HI – *Eco*RI fragment containing J<sub>H</sub>3 and J<sub>H</sub>4) kindly provided by Drs F. Young and F. Alt (The Children's Hospital, Boston, MA).

## Results

### Co-expression of endogenous $\mu$ and transgenic $\mu$ in hybridomas from M54 mice

Our previous FACS studies demonstrated three kinds of B cells in M54 mice: 'single-producers' that express transgenic IgM (Igh-6a, referred to hereafter as 6a); single-producers that express endogenous IgM (Igh-6b, referred to as 6b) alone; and 'double-producers' that co-express transgenic and endogenous (6a – 6b)  $\mu$  chains (14) (see, for example, Fig. 5 below). We also showed that sera from M54 mice contained 'mixed' IgM pentamers containing both 6a (transgenic) and 6b (endogenous)  $\mu$  chains in sera from M54 mice (14). Since hybridomas expressing such a double-producer phenotype had not been observed in previous studies of this strain we wished to directly examine whether hybridomas expressing both transgenic and endogenous  $\mu$  could be generated from M54 mice.

ELISA data, summarized in Table 1 and Fig. 1, show the  $\mu$

**Table 1.** Summary of M54 hybridoma fusions

Fusion no.	Strain	Wells with clones	IgM <sup>+</sup> wells	Wells containing IgM (Igh-6) allotype <sup>a</sup>			6a and 6b wells with mixed parameters number (%) <sup>b</sup>
				6a-only	6b-only	6a + 6b	
1	M54	130	77	18	36	23	NT
2	M54	114	73	11	15	47	NT
3	M54	117	72	20	28	24	24 (100)
4	M54	73	16	12	4	0	NT
5	M54	106	60	32	18	10	10 (100)
6	M54	123	35	20	10	5	5 (100)
7	M54	288 <sup>c</sup>	255	75	62	118	97 (82)
8	M54	111	63	38	16	9	9 (100)
9	C57BL/6	288 <sup>c</sup>	228	0	228	0	NT

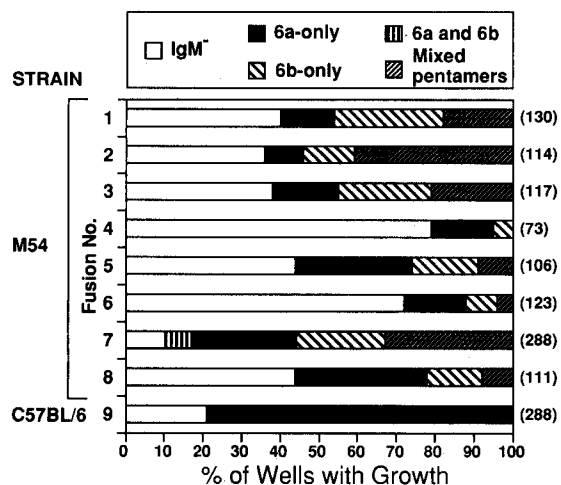
Mice were injected with 10  $\mu$ g LPS i.p. 2 days prior to fusion. Splenocytes from the mice were fused to SP2/0 myeloma cells at a 5:1 ratio and plated in 288 wells. Hybridomas were selected in HAT medium and screened for  $\mu$  heavy chain production via ELISA.

<sup>a</sup>IgM allotypes were assayed by coating ELISA plates with 331 (anti-IgM) antibody and revealing with either biotin – DS-1 (mouse anti-6a) antibody or biotin – AF6-78 (anti-Igh-6b) antibody followed by streptavidin – alkaline phosphatase.

<sup>b</sup>Wells positive for both 6a and 6b were tested for the presence of mixed (6a – 6b) molecules using a mixed sandwich assay in which ELISA plates were coated with anti-6a and revealed with <sup>b</sup>anti-6b. Value in parenthesis is the percentage of wells with mixed pentamers amongst total wells that test positive for both 6a and 6b. See text.

<sup>c</sup>Many wells contained multiple visible colonies.

NT = none tested.



**Fig. 1.** Summary of M54 and C57BL hybridoma fusions. A bar graph for each fusion is given showing the frequency of wells with hybridomas which are positive for no IgM (IgM<sup>-</sup>), 6a-only, 6b-only, both 6a and 6b or mixed pentamers. The value to the left gives the number of wells positive for hybridoma growth. See text for explanation.

heavy chain production obtained from hybridomas generated in eight independent fusions. Initial analysis of individual supernatants from the microtiter wells into which the fusion products (hybridomas) were distributed in each fusion reveals three kinds of wells, corresponding to the three kinds of B cells detected by FACS analysis: 6b only (endogenous  $\mu$ ); 6a only (transgenic  $\mu$ ); and a large number (14–64% of IgM<sup>+</sup> wells) of 6a–6b double-producers. *A priori* the presence of both 6a and 6b in supernatants from these latter, double-producing wells could also be explained by the presence of two hybridoma clones, one producing 6a and a second producing 6b. However, the frequency of double-producers is too high to be accounted for by the simple chance of two hybridomas in a single well. In all fusions but one (no. 7) hybridomas grew in only 35–40% of wells (indicating that the wells were generally seeded at <0.5 cells/well) and none of the wells observed visually shortly after seeding contained more than one colony. Under these conditions and assuming no double producers the frequency of wells with both a 6a and a 6b clone should be close to the product of the frequency of 6a-positive wells times the frequency of 6b-positive wells. This is clearly not the case for any of the fusions. For example in fusion 3 the frequency of 6a-positive is 0.15 (44/288) while the frequency of 6b-positive is 0.18 (52/288). Thus the expected frequency of wells with both 6a and 6b clones is 0.028 or approximately eight out of 288, in contrast to the observed frequency of 24 out of 288. As shown in the next section, the 6a–6b 'double' wells in these fusions contain hybridomas that produce mixed (6a–6b) IgM pentamers identical to those we previously characterized in the serum of M54 mice (14).

#### Many M54 hybridomas produce mixed (6a–6b) IgM pentamers

To directly assay for mixed IgM pentamers we use anti-6a and anti-6b anti-allotype antibodies in two versions of the 'mixed sandwich' ELISA that we developed earlier (14). We either coat with anti-6a and use anti-6b to reveal bound 6a-containing molecules that also contain 6b, or in the reverse direction we

**Table 2.** Specific detection of mixed IgM molecules produced by M54-derived hybridomas

Hybridoma supernatants	Coating antibody/revealing antibody (ELISA units/10)					
	$\alpha$ -IgM/ $\alpha$ -6a	$\alpha$ -IgM/ $\alpha$ -6b	$\alpha$ -6a/ $\alpha$ -6a	$\alpha$ -6b/ $\alpha$ -6b	$\alpha$ -6b/ $\alpha$ -6a	$\alpha$ -6a/ $\alpha$ -6b
6.1	225	444	191	435	482	196
6.3	460	434	441	383	543	325
7.2	399	501	228	370	564	235
8.1	261	376	369	259	466	130
Controls						
19.1.2 (6a)	469	12	517	0 <sup>a</sup>	0.5	4
2.31.1 (6b)	2	374	1	324	0 <sup>a</sup>	0 <sup>a</sup>
19.1.2 + 2.31.1 (6a + 6b)	341	346	518	346	5	0 <sup>a</sup>

Presence of mixed IgM molecules produced by double-producing M54 hybridomas was detected with various combinations of sandwich assays using different coating and revealing antibodies such as 331 [anti-IgM ( $\alpha$ -IgM)], DS-1 [anti-6a allotype ( $\alpha$ -6a)] and AF6-78.25 [anti-6b allotype ( $\alpha$ -6b)]. In all assays 50  $\mu$ l of supernatant was tested. Control mAb 19.1.2 and 2.31.1 were IgM anti-dextran antibodies derived from BALB/c (Igh-6a) and C57BL/6 (Igh-6b) mice, respectively. Readings, rounded to the nearest 10, were averages of duplicate wells and confirmed in a separate assay. Note that scores for positives and negatives are well separated. <sup>a</sup>ELISA readings were in the negative range with respect to blank.

coat with anti-6b and use anti-6a as the revealing reagent. In both cases a positive signal occurs *only* if the hybridoma product in a given well is recognized and bound by *both* anti-6a and anti-6b, i.e. if mixed IgM molecules that have both transgenic (6a) and endogenous (6b)  $\mu$  chains are present. Mixtures of homogeneous endogenous and transgenic IgM molecules do not score positive in either version of the mixed sandwich ELISA [see (14); Table 2; Table 1, fusion 7].

Data in Table 1 show that in all fusions except no. 7 mixed (6a–6b) molecules were detected in every well in which both 6a and 6b were initially detected in the single allotype assays (Tables 1 and 2). The conclusion that these are truly mixed molecules is reinforced by data from fusion 7, which was unusual in that all wells had hybridoma growth and visual observation revealed multiple colonies in the many of the wells. In this fusion, based on the frequency of 6a- and 6b-only wells, the expected number of wells with a 6a and a 6b clone should be approximately 16. Consistent with this, 21 wells were positive for Igh-6a and Igh-6b in the single allotype assays but were negative in the mixed sandwich assay. Thus in a fusion seeded such that two or more clones were likely to be present in a single well our assays readily detected and distinguished wells with a mixture of 6a and 6b antibodies, whereas in the sparsely-seeded fusions (1–6 and 8) that can be expected statistically to have only one clone per well, wells containing both 6a and 6b  $\mu$  chains all proved to contain mixed IgM molecules.

The findings presented above document the ability of the mixed sandwich assay to identify hybridoma clones producing mixed (6a–6b) IgM molecules; however, since the ability to recognize these mixed IgM pentamers has become an issue (27) we point out the following: (i) data in Table 2 show directly that a mixture of 6a and 6b molecules produced by separate hybridoma clones scores positive in the appropriate single-allotype ELISAs but does not score positive in either of the mixed sandwich assays; (ii)

mixed molecules are detectable irrespective of which anti-allotype antibody is used as the plate coat (capture) antibody and which is used as the revealing reagent; (iii) we have never observed a discrepancy between the two mixed sandwich assays; (iv) no supernatant which tested positive in the mixed sandwich assay has ever scored negative in the single anti-6a and anti-6b assays, e.g. supernatants from 20 wells (original and subcloned lines) identified as containing mixed molecules were positive for all the appropriate combinations of ELISA assays (Table 2 and data not shown); (v) there is no reduction in the binding activity in the mixed sandwich assay when supernatants scored as containing mixed IgM molecules are spun in an airfuge (15 min at 100,000 g) to remove aggregates of normal (non-hybrid) 6a and 6b IgM molecules that might have been present in the mixture (data not shown).

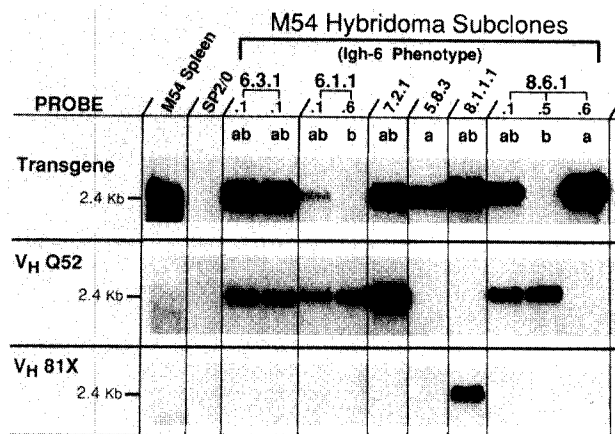
Finally, evidence presented below demonstrating that hybridomas which score as double-producers in the mixed sandwich ELISA have two mRNA species—one coding for the transgene and one for an endogenous gene—concludes the verification of this assay and provides unequivocal proof that double-producing hybridomas are readily obtained from fusions with M54 spleen cells (see Fig. 2). However, as data presented in the next section show, most of these double-producing clones are highly unstable and tend to convert to single producers very rapidly. This instability, we suggest, explains the failure to find double-producer hybridomas in the Weaver *et al.* study (8).

*Most double-producing hybridomas are unstable and convert rapidly to single-producers*

The data to this point have dealt with the detection of double-producing hybridomas in initial fusion wells (seeded just after fusion). To definitively characterize these double producing clones we were anxious to establish stable cloned sublines that would continue to produce mixed IgM molecules. Unfortunately, however, attempts at subcloning (by limiting dilution at <0.6 cells/well) demonstrated that the double-producers are extremely unstable. In fact most clones converted to single producers or became non-secretors within a single passage! Defining a stable clone as one in which >50% of the subclones retain the mixed phenotype, of 45 hybridoma wells that were originally identified as doubles and subcloned only six remained stable after two rounds of subcloning. Two of these were unstable during the third round of subcloning. In essence the aggregate of our experience demonstrated that unlike most hybridomas, which become stable after the first or second round of subcloning, the M54 double-producers remain highly unstable through at least four rounds of subcloning, making it very difficult to preserve the double producing lines.

Table 3 shows some of the clones of M54 hybridomas and the results of subsequent rounds of subcloning. In each case the digit in the clone name that precedes the first decimal point represents the fusion number. The next digit represents the initial well designation and all following numbers note individual subclones. Thus clone 8.6.15 has been subcloned twice. Although some hybridomas, such as 6.3 and 7.2, remained double-producing through 2 rounds of subclonings, most clones, typified by 1.7, 6.1, 7.5 and 8.6, yielded a substantial portion of single-producing or non-secreting subclones with each round of subcloning.

To ensure that any double-producing hybridoma lines that we



**Fig. 2.** Loss of endogenous or transgene mRNA in subclones correlates with the phenotype as measured by ELISA. Total cytoplasmic RNA was isolated from M54 mouse spleen, SP2/0 myeloma cells and M54 hybridomas by the LiCl-urea method. RNA (10  $\mu$ g) was loaded in each lane and electrophoresed in a 1% agarose-formaldehyde gel. Lane 1: M54 mouse spleen; lane 2: SP2/0; lanes 3-12: M54 hybridoma subclones as marked. The labels below the clone number give the phenotype a, b, or a/b as measured by ELISA. Gel was hybridized with random-primed [<sup>32</sup>P]dATP-labeled 17.2.25 m (*EcoRV-StuI* fragment) [A]; random-primed [<sup>32</sup>P]dCTP-labeled V<sub>H</sub>81X [B]; random-primed [<sup>32</sup>P]dCTP-labeled V<sub>H</sub>Q52 [C] probes, respectively. Between each hybridization the gel was stripped using standard low salt conditions.

established were indeed clonal we performed a third round of subcloning, in which we again observed frequent conversions from the double-producer parent to single-producers or non-secretor progeny clones. Interestingly, we noted that the progeny of a given initial clone often tend to lose the ability to produce the same IgM. For example subclones of 1.1 and 8.6 tend to stop producing the endogenously encoded IgM and thus predominantly become 'transgene-only' clones, while subclones of 1.7 and 6.1 tend to lose production of the transgene and thus mainly become 'endogenous-only' clones.

The instability of the double-producers proved to be quite striking. In essence we found that in order to maintain hybridomas that continue to produce mixed molecules they must be continuously subcloned and selected for this property (at least through four subclonings). Even detecting double-producers in the initial wells shortly after fusion requires care. When a series of the original double-positive fusion wells were routinely aspirated and fed with new media the mixed molecules were diluted out and the wells eventually scored as a single positives (data not shown).

Given that in our studies the average percentage of Ig<sup>+</sup> wells which were mixed pentamers was 30% (range: <6-64%) (Table 1) and approximately 10% of these remained stable upon subcloning, only 1-3 wells in 100 would appear to give double-producers and even these would appear to be 'suspect' since they would continue to throw off singles. Thus, it is very likely that these highly unstable double-producing hybridomas, which were not expected in the Weaver studies, were missed due to the rapid conversion to single producers.

Since we always scored the original media (supernatant) from the fusion wells the frequency of double-producing hybridomas

**Table 3.** Most of Igh-6a/6b 'double-producer' hybridomas convert to single-producers in successive clonings

Original well	Subclonings														
	Round 1					Round 2					Round 3				
	No. tested	Igh-6a/b <sup>+</sup>	Igh-6a <sup>+</sup>	Igh-6b <sup>+</sup>	Neg.	No. tested	Igh-6a/b <sup>+</sup>	Igh-6a <sup>+</sup>	Igh-6b <sup>+</sup>	Neg.	No. tested	Igh-6a/b <sup>+</sup>	Igh-6a <sup>+</sup>	Igh-6b <sup>+</sup>	Neg.
1.1	23	14	9	0	0	24	1	23	0	0					
						21	1	20	0	0					
1.2	12	10	0	2	0	25	0	0	24	1					
						5	0	0	5	0					
						30	0	0	29	1					
1.7	24	4	10	9	1	12	0	12	0	0					
						10	2	6	2	0	19	0	19	0	0
											23	0	23	0	0
1.8	40	34	6	0	0	26	24	0	0	2					
6.1	57	52	0	5	0	25	10	0	15	0	30	0	0	28	2
6.3	34	34	0	0	0	18	17	0	0	1	35	33	2	0	0
7.2	31	29	0	2	0	53	53	0	0	0					
7.4	13	6	2	2	3	38	38	0	0	0	15	0	0	15	0
						8	7	0	1	0					
7.5	38	4	2	4	28	21	0	0	0	21					
8.1	57	54	0	3	0	9	9	0	0	0	13	9	1	0	3
8.6	25	3	20	1	1	35	19	12	4	0	16	8	3	1	4
						26	0	26	0	0					
8.8	41	3	26	0	12	21	0	1	3	17					

Hybridoma wells that were positive for both a and b allotypes were subcloned by limiting dilution to a final concentration of 50–60 cells/96-well plate. Subclones were tested via ELISA as in Table 1 and subclones positive for both 6a and 6b were subjected to subsequent rounds of subcloning. Each line in each round represents the subcloning of a single well. For example, from the original well 8.6, three of 25 subclones tested were 6a–6b doubles. Two of these double clones, 8.6.1 and 8.6.2, were expanded and subcloned, yielding 19 and 0 6a–6b double clones respectively. One of the double subclones of 8.6.1 (.1) was subcloned for a third round.

reported here most accurately reflects their true frequency amongst the hybridomas generated from the fusion; however, even these values must be taken as a minimum since some of the doubles may have converted to single producers before detectable amounts of mixed IgM had been produced.

#### *Anti-Ig binding by M54 endogenous IgM does not account for the double-producers*

To explain our findings, Imanishi-Kari and colleagues (27) have suggested that many of the endogenous (Igh-6b) antibodies in the transgenic mice are low affinity poly-reactive antibodies that can bind the DS-1 anti-6a antibody used to reveal the transgenic  $\mu$ . According to this hypothesis the cross-reactive specificity of these antibodies would make it appear that a population of M54 B cells expressing only endogenous (6b) Ig also express transgenic IgM (6a) (that is bind anti-6a). Similarly, antibodies produced by these cells would be detected as mixed (6a–6b) pentamers.

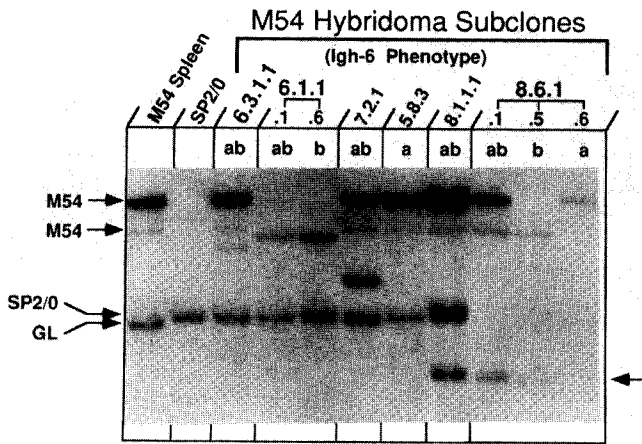
This (remote) possibility is ruled out by data from these hybridoma studies. First, as we have shown, double-producers routinely convert to single-producer clones. If these doubles were due to endogenous (6b-only) antibodies cross-reacting with the anti-6a monoclonal reagent both the anti-6a and the anti-6b reactivities would have been lost simultaneously, i.e. the double-producers could neither become single 6a producers nor single 6b producers. Secondly, the endogenous IgM produced by the double-producing hybridomas does not appear to have significant anti-idiotype or anti-Ig binding activity since 25 of 25 double producers tested showed no capacity to bind to either mouse or goat IgG in ELISA assays (data not shown). These data, together with evidence presented in the next section, definitively

lay to rest arguments based on the idea that the double producing hybridomas (and B cells) that we detect are actually producing a single species of cross-reactive, endogenously encoded IgM.

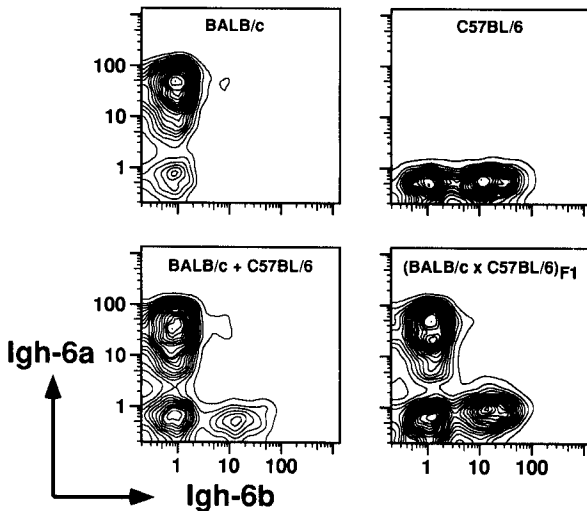
#### *Double-producing hybridomas have mRNAs encoding both transgenic and endogenous $\mu$ , single-producing subclones lack mRNA coding for the missing $\mu$ chain*

To verify the co-expression of transgenic and endogenous  $\mu$  heavy chains in the M54 hybridomas clones were probed for the presence of transgene encoded and endogenously encoded Ig mRNA by Northern analysis. We tested a set of six clones derived from independent double-producers from four different fusions. Five of the clones selected were double-producers and one produced only the transgene  $\mu$ . In addition we tested a series of subclones derived from three of the double-producing clones. As Fig. 2 shows, all six clones tested were producing the transgene encoded  $\mu$  chain (detected by ELISA) and all six showed a clear mRNA band when blotted with a probe specific for the  $\mu$  transgene. Similarly, all subclones that continued to produce the transgene Ig showed a clear mRNA band with the transgene probe. Those subclones (6.1.1.6, 8.6.1.5) that no longer produce the transgene Ig did not have the transgene mRNA. Thus detection of transgene encoded  $\mu$  in ELISA assays always corresponds to detection of intracellular messenger RNA encoding this molecule.

Similarly, detection of endogenously encoded  $\mu$  mRNA always corresponds to detection of endogenously encoded  $\mu$  protein in the ELISA assay (Fig. 2). All of the clones and subclones that typed as doubles or 6b-only had messenger RNA coding for an endogenous  $\mu$  heavy chain. In four of six initial clones (and their progeny) this message was detectable with a probe specific for



**Fig. 3.** Southern blot analysis of double-producing hybridomas from M54  $\mu$  heavy chain transgenic mice. DNA were isolated from M54 mouse tissues and hybridomas, digested with the restriction enzyme *Sma*I and electrophoresed in a 0.8% agarose gel. DNA (10  $\mu$ g) were loaded in each lane and hybridized with random-primed [<sup>32</sup>P]dCTP-labeled J<sub>H</sub> probe (a 2 kb *Eco*RI – *Bam*HI fragment containing J<sub>H</sub>3 and J<sub>H</sub>4). Lane 1: M54 mouse spleen; lane 2: SP2/0; lanes 3 – 12: M54 hybridoma subclones as marked. Arrows indicate the: germline J<sub>H</sub> band (GL); transgene specific bands (M54); SP2/0 specific band (SP2/0). The arrow on the right side marks the rearranged band for the 8.6.1 clones.



**Fig. 4.** Specificity controls for anti-6a and anti-6b reagents used in flow cytometry. Peritoneal cells from BALB/c, C57BL/6, (BALB/c x C57BL/6) F<sub>1</sub> mice or a mixture of BALB/c and C57BL/6 [BALB/c + C57BL/6] were stained with <sup>F1</sup>anti-Igh-6a (DS-1) and <sup>B1</sup>anti-Igh-6b (AF6-78). Biotin conjugates were revealed with Texas red – avidin. The ratio of 6a<sup>+</sup> to 6b<sup>+</sup> cells in the mixed sample is skewed because the frequency of B cells was higher in the BALB/c mice. The cells shown have been gated for lymphocytes by forward and side scatter.

the V<sub>H</sub>Q52 V<sub>H</sub> gene family and the remaining clone with a probe specific for the V<sub>H</sub>81X gene family. The preferential use of these J<sub>H</sub> proximal gene families in hybridomas from  $\mu$  transgenic mice has been previously reported (8,27,28); the V<sub>H</sub> gene utilization that we observe supports the conclusion of these earlier studies indicating that the presence of the transgene seriously perturbs the V<sub>H</sub> gene repertoire in M54 mice.

Analysis of the mRNA expressed in subclones derived from double-producers demonstrates that loss of the ability to produce either the transgenic or the endogenous  $\mu$  is always reflected in the loss of the corresponding mRNA band (see Fig. 2). For example we examined three subclones of the double-producing clone 8.6.1: the subclone that retained the double-producing phenotype (8.6.1.1) continued to express both the transgene and the endogenous mRNAs; the subclone that converted to transgene-only phenotype (8.6.1.6) retained the transgene mRNA band and lost the endogenous band; the subclone that converted to endogenous-only phenotype (8.6.1.5) retained the endogenous message and lost the transgene mRNA. Thus the presence of transgenic and/or endogenous  $\mu$  mRNA invariably correlates with the  $\mu$  heavy chain produced by the subclone (as determined by the various ELISA assays).

Southern blot analysis with a J<sub>H</sub> specific probe confirms the presence of IgH rearrangements in the hybridomas discussed above and documents the parent/progeny relationships of the 6.1.1 and 8.6.1. subclone families discussed above (see Fig. 3). In addition, data from this analysis suggest that the loss of endogenous  $\mu$  production is due to the loss of the rearranged IgH gene in the clone while loss of transgene  $\mu$  production is associated with the loss of part (but not all) of the transgene complex, i.e. the dominant 12.8 kb transgene band.

The 4.5 kb band contributed by SP2/0 proved particularly useful as a clonal marker in this study. This band is visible in all

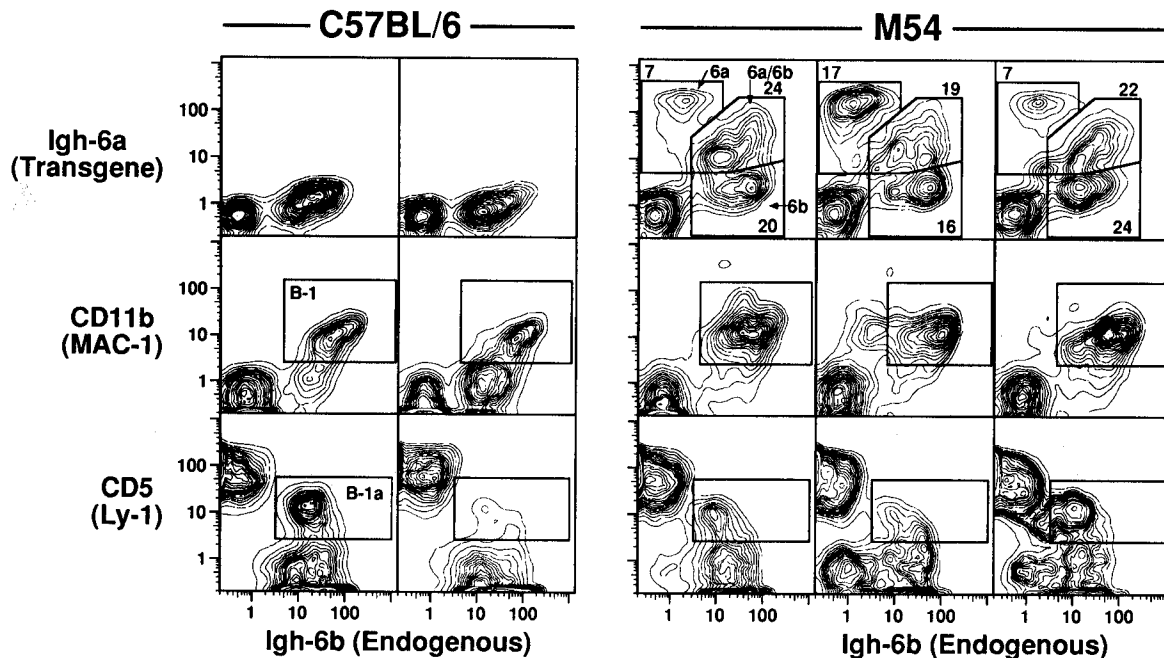
of the hybridoma clones and subclones tested except for subclones derived from 8.6.1. The three subclones derived from this parent clone have lost this SP2/0 band (see Fig. 3), confirming that these are indeed derived from the same (double-producing) parental hybridoma.

*Peritoneal cavity B cells expressing endogenous  $\mu$  in M54 mice belong to the B-1 lineage*

The detection of single- and double-producing hybridomas is consistent with data from our previous FACS studies of splenic and peritoneal B cells. As in the ELISA assays, Fig. 4 demonstrates that specificity of the anti-6a and anti-6b reagents. No significant staining of the inappropriate allotype (i.e. double-positive cells) is detected in the BALB/c, C57BL/6 or mixed spleen cells and no true doubles are seen in the F<sub>1</sub> mice. Note, however, that there is a very slight but detectable background staining of the opposite allotype in the F<sub>1</sub> sample (compare the Igh-6a staining of the Igh-6b positive cells in the F<sub>1</sub> and C57BL/6 mice).

The typical FACS analysis of peritoneal cells from M54 and control C57BL/6 mice shown in Fig. 5 confirms that M54 has three distinct B cell populations: 6a only; 6b only; and double-expressing B cells that stain strongly with both anti-6a and anti-6b. In the M54 mice, as is seen in allotype heterozygous F<sub>1</sub> mice, what we define as the two single-expressing populations stain dully for the opposite allotype. This staining may be due to naturally occurring V<sub>H</sub>81X B cell specific autoantibodies, which have been described by Kearney (29).

Table 4 summarizes the relative frequencies of these populations (as gated in Fig. 5) in the spleen and peritoneal cavity (PerC) of normal and M54 mice. In our earlier study we reported that in M54 mice approximately 50% of the splenic and 90% of the PerC B cells express endogenous  $\mu$  (14); however, others have reported significantly lower values (27). These data show that



**Fig. 5.** In M54 peritoneum the endogenous  $\mu^+$  B cells belong to the B-1 cell lineage. Peritoneal cells from M54 and control C57BL siblings were stained with  $F_{12}$ anti-Igh-6a (DS-1),  $B_{12}$ anti-Igh-6b (AF6-78) and  $A_{PC}$ anti-CD5 (53-7) or with  $F_{12}$ anti-Igh-6b and  $A_{PC}$ anti-CD5. Biotin conjugates were revealed with Texas Red – avidin. Data is shown as 5% probability contour maps. The gates used to calculate the values of the 6a-only, 6b-only and 6a – 6b doubles (summarized in Table 4) are shown in the top panels. The values for percent of lymphocytes are shown for each of the populations. Gates for B-1 cells (middle panels) and B-1a cells (bottom panels) are shown.

**Table 4.** Lymphocyte populations expressing transgenic and endogenous IgM varies with age in M54 heavy chain transgenic mice

Mouse strain	Age (months)	n	Total lymphocytes ( $\times 10^6$ )	Lymphocytes (%)		
				6a-only	6b-only	6a – 6b
<b>Spleen</b>						
M54	6–8	6	28 $\pm$ 0.6	18 $\pm$ 4.3	0.8 $\pm$ 0.4	3.3 $\pm$ 0.8
M54	15–17	5	22 $\pm$ 0.4	8 $\pm$ 2.3	6.2 $\pm$ 2.3	3.5 $\pm$ 0.7
C57BL/6	6–8	5	48 $\pm$ 1.6	<0.1	52 $\pm$ 7.0	<0.2
<b>PerC</b>						
M54	6–8	5	2.9 $\pm$ 1.3	22 $\pm$ 4.8	12 $\pm$ 2.8	20 $\pm$ 6.1
M54	15–17	5	3.0 $\pm$ 0.8	6.9 $\pm$ 2.3	23 $\pm$ 5.3	26 $\pm$ 5.2
C57BL/6	6–8	4	8.9 $\pm$ 0.8	<0.1	65 $\pm$ 8.0	<0.4

Cell suspensions from C57BL/6 and M54 mice were stained with  $F_{12}$ anti-6a,  $B_{12}$ anti-6b and  $A_{PC}$ anti-MAC-1 or  $A_{PC}$ anti-CD5. Biotinylated antibodies were revealed by incubation with Texas Red – avidin. Lymphocytes in each population were determined as a percentage  $\pm$  SD of total cells within lymphoid (forward versus orthogonal scatter) profiles.

this difference is due to the age of the mice used in the respective studies, i.e. 9–12 month old mice were used in our study while 2–6 month old mice were used by Iacomini *et al* (15). Table 4 shows that cells expressing endogenous IgM constitute progressively higher percentages of the Ig $^+$  cells as the animals age.

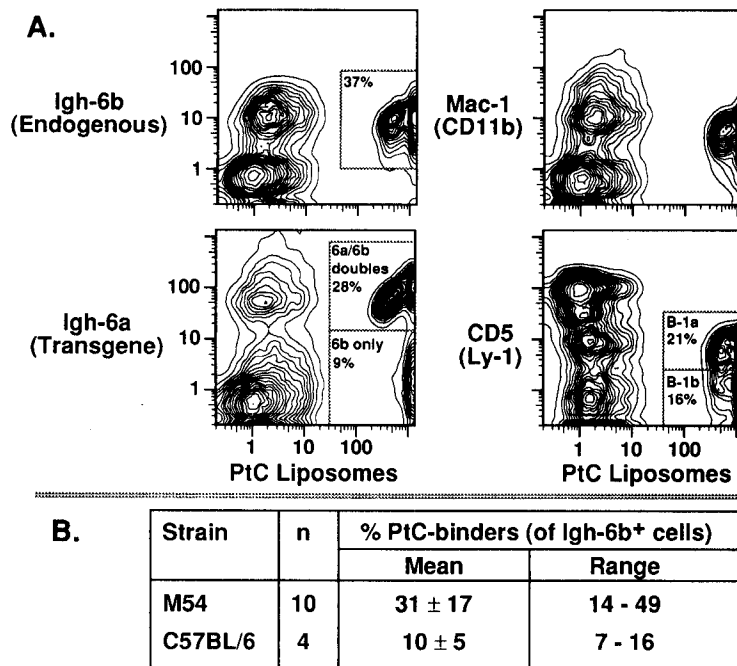
Characterization of the cells expressing endogenous  $\mu$  indicates that these cells belong largely or exclusively to the B-1 lineage (Fig. 5). Previous studies have shown that murine B cells are divided into two overall lineages that derive from distinct progenitors (30–32). Conventional B cells, which constitute the predominant population in the spleen, are derived *de novo* throughout life from progenitors in the bone marrow; B-1 (Ly-1

B) cells, which predominate in the PerC, differentiate *de novo* early in life but persist via self replenishment in adults (10,33).

B-1 cells are readily distinguished in the PerC where they constitute the major B cell population, expressing relatively high levels of IgM, expressing low levels of IgD and MAC-1 (CD11b) and are sub-divided by the expression of CD5 (Ly-1). CD5 $^+$  B-1 cells (designated B-1a) and CD5 $^-$  B-1 cells (designated B-1b) (11) have been shown to be distinct, yet closely related, B cell lineages (31). Conventional B cells are also present in the PerC, however they express high levels of IgD, low levels of IgM and do not express either CD5 or MAC-1.

Based upon their characteristic expression of MAC-1, greater than 85% of the M54 PerC B cells that express endogenous  $\mu$





**Fig. 6.** Anti-phosphatidylcholine-binding B cells in the PerC are endogenous (Igh-6b) positive B-1 cells. (A) M54 peritoneal cells were stained with <sup>125</sup>I-PtC liposomes, <sup>B1</sup>anti-6b and one of the following: <sup>APC</sup>anti-6a, <sup>APC</sup>anti-MAC-1 or <sup>APC</sup>anti-CD5. Data is presented as in Fig. 3. The percentages given represent the percent of Igh-6b+ cells which fall within the box. No PtC liposome binders were ever seen in the 6a-only population. (B) The frequency of PtC liposome binders in normal C57BL/6 and M54 mice.

(alone or in conjunction with transgenic  $\mu$ ) clearly belong to the B-1 lineage (see Fig. 5). This is in fact a conservatively low estimate given the low expression of the MAC-1 antigen. The remaining 15%, while falling into the region of the background, also appear to express very low levels of MAC-1 (compare the contour lines of C57BL/6 to M54 in the middle panels). On average 30% (range 14–60%) of these endogenous  $\mu^+$  B-1 cells are B-1a (CD5+) cells; the remainder are B-1b (CD5-) cells. Double-expressing B cells are found in both the B-1a and B-1b populations at frequencies that vary from animal to animal. In addition a population of B-1b cells that express only transgenic  $\mu$  is detectable in some animals. Like M54 PerC, M54 spleen contains some CD5+ B-1a cells that express endogenous  $\mu$  alone or in combination with transgenic  $\mu$ ; however, limitations in distinguishing conventional B cells from B-1b cells in spleen (because splenic B-1 cells do not express MAC-1) make it difficult to accurately determine whether all of the cells that express endogenous  $\mu$  belong to the B-1 lineage.

The conventional B cells (CD5-, MAC-1-) in M54 PerC are relatively rare in comparison with the distinct conventional B cell population detectable in C57BL/6 control mice (see Fig. 5). FACS analyses show that all of the M54 conventional B cells in PerC express transgenic  $\mu$ ; none express endogenous  $\mu$ . Thus the M54 PerC conventional B cell population is composed exclusively of transgene-only cells. M54 spleen also appears to contain a sizable number of these transgene-only conventional B cells (14), however, as indicated above, the lack of a definitive marker to distinguish conventional from B-1b cells in spleen makes it difficult to determine whether any of the splenic double-producers or endogenous-only B cells belong to the conventional lineage.

*The peritoneal B-1 repertoire is altered in M54 mice*

The functional repertoire of the two B cell lineages are distinct. B-1 cells produce many natural antibodies, polyreactive antibodies, autoantibodies and antibacterial antibodies (e.g. the autoantibody response to PtC and the responses of phosphorylcholine and dextran), but do not respond to most protein antigens. Conventional B cells, in contrast, produce most of these latter responses and appear to produce certain T-independent responses as well (10,33–37).

Weaver *et al.* (8) and others have shown that the presence of the M54 transgene perturbed  $V_H$  gene usage and resulted in an over utilization of the  $V_H81X$  and  $V_HQ52$  families in B cells. As indicated earlier the  $V_H$  gene usage in the hybridomas that we generated in this study confirm this finding. They suggested that this alteration in repertoire could be due to the expression of the transgene early in ontogeny resulting in the over-expression of  $V_H$  genes which mimic the idiotype of the introduced transgene. Alternatively, given the differences between the functional repertoires of B-1 cells and conventional B cells, the spectrum of  $V_H$  genes in the M54 mice may just reflect the lack of conventional B cells expressing endogenous  $\mu$  in these mice.

To examine whether there were also perturbations within the B-1 repertoire in M54 mice we used FACS analyses that reveal cells producing the anti-PtC antibodies mentioned above. These cells, which bind fluorochrome-labeled liposomes containing membrane PtC (Fig. 6), constitute a major component of the B-1 repertoire but are not detectable amongst conventional B cells (38). Consistent with this, all of the PtC-binding B cells in M54 PerC are found in the B-1 population, either as B-1a (CD5+,

Mac-1<sup>+</sup>) or B-1b (CD5<sup>-</sup>, Mac-1<sup>+</sup>) cells (Fig. 6). However, whereas PtC-binding cells normally comprise approximately 10% of all peritoneal B-1 cells in C57BL/6 mice, their frequency in M54 mice varies dramatically from animal to animal (range 14–49%) averaging 30% of the PerC B cells that express endogenous IgM (see, for example, Fig. 6). Thus the altered V gene usage that Weaver *et al.* detected in hybridomas derived from M54 mice is similarly reflected in changes in the expressed repertoire of the peritoneal B-1 lineage cells in these animals.

Curiously, the repertoire alterations observed in M54 PerC and spleen differ. The anti-PtC that represent a sizable proportion of the B-1 cells in PerC utilize the V<sub>H</sub>11 and V<sub>H</sub>12 gene families (39). However, the M54 hybridomas that we and Weaver *et al.* derived from spleen cell fusions display a heavy preference for the V<sub>H</sub>Q52 and V<sub>H</sub>81X gene families. This may reflect a different repertoire in the B cells found in these two sources, since anti-PtC B cells represent less than 1% of the B cells in the spleens of M54 mice.

## Discussion

In every  $\mu$  transgenic mouse we have analyzed by flow cytometry we have identified B cells co-expressing transgenic and endogenous  $\mu$  (14,40). However, in the studies of Weaver *et al.* (8) hybridomas which simultaneously produced both chains were not observed, leading to the suggestion that the double-producers were an artifact. To address this apparent conflict we generated a series of hybridomas from M54 mice. Analysis of these hybridomas confirm much of the essential findings reported by Weaver *et al.* and reconciles their findings with apparent conflicts that emerged from our later studies with the M54 transgenic mice.

Molecular and serological analysis of these hybridomas presented here clearly demonstrate that the double-producers are not a serological artifact but represent a unique population of cells present in M54 and in other  $\mu$  transgenic strains. However, the 'double-producing' hybridomas generated from M54 mice are so unstable that they almost always convert to single-producers or non-producers by the time subcloned hybridoma lines are established, even when carefully monitored in an attempt to rescue stable 'double-producing' clones. Thus it is not surprising that the hybridomas established by Weaver *et al.* were singles producing either transgenic or endogenous IgM, not both (8).

While we did not attempt to reproduce all of the studies of Weaver *et al.*, our data are consistent with a number of their observations. They demonstrated that a high proportion of hybridomas recovered from M54 produce IgG. Consistent with this we found that the initial wells in the one fusion that we examined for this purpose contained a high proportion of hybridomas (20%) expressing  $\kappa$  but not  $\mu$  (data not shown). Similarly, initial studies by Weaver *et al.* (8), extended by Iacomini *et al.* (27), demonstrated that hybridomas recovered from M54 mice disproportionately express V<sub>H</sub> genes from the proximal (V<sub>H</sub>Q52 and V<sub>H</sub>81X) V<sub>H</sub> families. To the extent that hybridomas can be used as an index of the 'parental' B cell repertoire we also found significant skewing in the Ig repertoire expressed in M54 mice in that four of six hybridomas tested expressed V<sub>H</sub> genes from these families. In addition we show that this alteration

extends to the peritoneal B-1 cell repertoire as evidenced by a 3-fold increase in the frequency of B-1 cells specific for PtC.

We specifically did not investigate the *in situ* idiotype repertoire expressed in M54 mice for three reasons. First, the J<sub>H</sub> proximal V<sub>H</sub> gene family that the hybridoma data suggest are expressed in these mice are now known to code for Ig molecules that frequently react with a variety of antigens, including other Ig molecules, and could be expected to yield confusing results with the conventional guinea-pig anti-idiotype sera used in the earlier work. Secondly, M54 mice have sizable numbers of plasma cells that produce both transgenic and endogenous IgM. This as we have shown results in the secretion of 'mixed' pentamers that are readily detectable in sera and would confuse anti-idiotype studies of the sera. (The assays used in the Weaver *et al.* studies were not designed to detect these molecules, which at the time were not thought to exist.) Third, there are substantial technical difficulties associated with the use of guinea-pig anti-idiotype antibodies such as those used in the earlier studies.

Finally, the shifts in the repertoire such as were observed by Weaver *et al.* can be explained by the alteration in the relative frequency of the B-1 and conventional B cell lineages in these mice and need not invoke idiotypic mimicry. A number of studies indicate that B-1 cells, which are the predominant population during fetal development, have a distinctive repertoire which is skewed towards a fetal profile (i.e. an over-representation of the J<sub>H</sub> proximal V<sub>H</sub> families, 81X and Q52) (12,13). Thus the altered repertoire in these mice characterized by the over-expression of V<sub>H</sub>81X and V<sub>H</sub>Q52 can be simply the result of the 'fetal-like' repertoire of the endogenous  $\mu$  produced almost exclusively by the B-1 cells.

We have previously shown (i) that conventional B cell development in M54 mice is severely inhibited (9) and (ii) that adoptive transfer of M54 bone marrow results in the predominant reconstitution of conventional B cells which are transgene only (40). In this paper analysis of M54 peritoneal cells shows that essentially all of the B cells expressing endogenous  $\mu$  belong to the B-1 lineage (Fig. 5). Taken together these data show that B cell differentiation is severely altered in M54 mice, resulting in the loss of a functional conventional B cell population capable of expressing endogenous Ig molecules and the predominance of a B-1 population which can express endogenous Ig but whose repertoire is known to be restricted and highly sensitive to selective pressures.

The alterations in B cell development and endogenous  $\mu$  gene expression which we have documented in the M54 strain is not unique to this strain or transgene. Double-producers have been identified by FACS in a large proportion of  $\mu$  transgenic mice examined (5,28,40,41). Similarly, the simultaneously expression of endogenous Ig and transgene IgM have since been documented in the serum of Sp6 mice expressing anti-TNP heavy and light chains (42) and 243-4 mice (43) expressing a M167 anti-phosphorylcholine  $\mu$  transgene (J. J. Kenny, personal communication). Finally, analogous to the studies presented here, Kroese and Kantor have shown that in the Sp6 strain the endogenous  $\mu$  protein is made almost exclusively from the B-1 population (F. G. M. Kroese, personal communication). Thus the defect in conventional B cell development and the preferential expression of the endogenous  $\mu$  gene by B-1 cells is not dependent on insertional effects or the specificity of the transgene but rather reflects the common effect of the introduction of any functionally

rearranged *μ* transgene in conventional B and B-1 cell development.

Interestingly, Kitamura *et al.* (44) have shown that, similar to *μ* transgenic mice, mice lacking  $\lambda 5$  and thus presumably unable to express the newly rearranged membrane *μ* in pre-B cells lack most conventional B cells but develop normal numbers of B-1 cells. Thus the expression of a *μ* membrane complex may be critical to the development of conventional B cells but not to the development of B-1 cells. Taken together these findings suggest sharp developmental differences between B-1 and conventional B cells, including the importance of the expression of membrane IgM in signaling the termination of IgH rearrangement and the onset of differentiation events that result in the development of mature conventional B cells.

Similarly, in *μ* transgenic mice inappropriately premature expression of membrane *μ* in conventional B cells may selectively block IgH rearrangement while, in a small number of cells, allowing development to maturity without IgH rearrangement. (The inefficiency of this process could indicate that the *μ*- $\lambda 5$  complex may actually be required for triggering further conventional B cell development.) Thus data from both studies are consistent with the model that pre-B cells in the conventional lineage are preferentially sensitive to signals from surface *μ* molecules that are unnecessary to go unheeded in the differentiation of B-1 pre-B cells to mature B cells.

B-1 cells are similar developmentally to avian B cells whereas conventional B cells represent a more highly evolved mammalian form (45). That B cell lineages that appear to have evolved at substantially different times have markedly different developmental mechanisms is not particularly surprising. These and other studies suggest that the development of this 'more advanced' conventional lineage can be altered/blocked by a variety of conditions (i.e. lack of  $\lambda 5$ , temporally inappropriate expression of rearranged *μ* heavy chain) that do not interfere with the development of the more 'primitive' lineage. Recognition of these differences is crucial to studies of B cell development and an understanding of the roles of the B cell lineages in the immune system.

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**Abbreviations**

6a	Igh-6a, 'a' allotype <i>μ</i> heavy chain
6b	Igh-6b, 'b' allotype <i>μ</i> heavy chain
6a-6b	mixed IgM molecules containing a and b allotype <i>μ</i> chains
APC	allophycocyanin
Bi	biotin
Fl	fluorescein
PE	phycoerythrin
PerC	peritoneal cavity
PtC	phosphatidylcholine

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#### Note added in proof

Since the submission of this paper, Imanishi-Kari and colleagues have published two papers on  $\mu$  gene expression in M54 mice (1,2). In the first paper they interpret their data as indicating that hybridomas expressing endogenous  $\mu$  exclude expression of transgene  $\mu$  and that the few double-producing hybridomas that they now find are due to the presence of two independent cell lines. The results in this paper clearly demonstrate that this interpretation is not valid for the numerous true double-producers that we have previously identified in M54 mice nor for the double-producing M54 hybridomas that we have characterized here. The second paper details examples of mAbs derived from M54 hybridomas with apparent anti-idiotypic reactivity and suggests that such antibodies are responsible for the double-producers that we and other labs have documented in numerous strains of  $\mu$  transgenic mice. Again, while such antibodies may exist within the M54 mice, our data definitively demonstrate that they are exceptions and cannot account for a significant portion of the double-producers (cells or hybridomas) which we have documented in these mice.

- 1 Imanishi-Kari, T., Bandyopadhyay, S., Busto, P., and Huang, C. A. 1993. Endogenous Ig production in  $\mu$  transgenic mice. I. Allelic exclusion at the level of expression. *J. Immunol.* 150:3311.
- 2 Imanishi-Kari, T., Huang, C. A., Iacomini, J., and Yannoutsos, N. 1993. Endogenous Ig production in  $\mu$  transgenic mice. II. Anti-Ig reactivity and apparent double allotype expression. *J. Immunol.* 150:3327.