LAH367

International Immunology, Vol. 5, No. 10, pp. 1317–1327

Evidence that intestinal IgA plasma cells in μ,κ transgenic mice are derived from B-1 (Ly-1 B) cells

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Key words: B-1 cells, CD5 B cells, FACS, IgA plasma cells, Ly-1 B cells, mucosal immunity, transgenic mice

Abstract

B6-Sp6 transgenic mice carry fully rearranged (BALB/c-derived, Igh-C^a allotype) μ heavy chain and κ light chain transgenes, specific for trinitrophenyl, on a C57BL background (Igh-C^b allotype). FACS analyses show that the majority of B cells in peripheral lymphoid organs and bone marrow (BM) express transgenic IgM exclusively. A small proportion of the B cells, however, express endogenous IgM, usually concomitant with transgenic IgM. Three criteria establish that the endogenous IgM expressing B cells belong to the B-1 cell lineage. (i) Endogenous IgM expressing B cells in B6-Sp6 mice have the same localization pattern as B-1 cells from normal animals: they are enriched in the peritoneal cavity. (ii) The endogenous IgM$^+$ B cells have the phenotype of B-1 cells: the endogenous IgM$^+$ peritoneal B cells express Mac-1 (CD11b) and low levels of IgD, and most also express CD5 (Ly-1). (iii) B6-Sp6 BM poorly reconstitutes endogenous IgM$^+$ B cells, just as adult BM from normal mice poorly reconstitutes B-1 cells. In contrast, B cells which only express the transgene are readily reconstituted by B6-Sp6 BM. The few endogenous IgM$^+$ cells in the B6-Sp6 BM recipients are located in the peritoneal cavity and have the phenotype of B-1b cells (previously the Ly-1 B sister population), which are known to be reconstituted by adult BM. Two-color immunofluorescence staining of tissue sections from the gut and from isolated gut lamina propria cells shows the presence of many IgA containing cells, about one-third of which simultaneously express cytoplasmic (transgenic) IgM. The C-region of this IgA is produced by endogenous C_α genes, because the transgene encodes only for C_μ. Furthermore, the majority of gut IgA containing cells do not express the idiotype of the transgene, indicating that most of the gut IgA cells are encoded by endogenous V_H genes and thus the result of an isotype switch from endogenous IgM expressing B cells. Since the endogenous IgM$^+$ cells are B-1 cells (both B-1a and B-1b), the data strongly indicate that the intestinal IgA plasma cells also belong to the B-1 cell lineage.

Introduction

IgA plasma cells in the intestine had been generally believed to be derived from precursor cells located in Peyer's patches (PP) (for reviews, see 1 and 2). According to this view, the vast majority of these IgA precursor cells are IgM and IgD bearing cells. These precursor cells are triggered by antigen derived from the gut, leave the PP, migrate to mesenteric lymph nodes (LN), and then enter the circulation via the thoracic duct. From the blood these cells migrate to the gut lamina propria (and other mucosal tissues) to differentiate into IgA producing plasma cells.

We have recently shown (3) that this view may be correct for only some of the IgA producing plasma cells in the gut; a significant proportion of these cells appear to derive from B-1 cells, which were formerly known as Ly-1 B cells (4). B-1 cells differ in anatomical localization, functional characteristics, and

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Transmitting editor: A. Radbruch

Received 7 January 1993, *accepted 8 June 1993*

gene expression from conventional B cells (for reviews, see 5 – 8). B-1 cells predominate in peritoneal and pleural cavities, are self-replenishing and arise from distinct progenitors (9 – 13). Importantly, B-1 cells also differ in their antibody repertoire; they produce 'natural antibodies' and antibodies directed to autoantigens and bacteria-related antigens (14 – 19).

A role for B-1 cells in the generation of IgA plasma cells in the gut lamina propria was suggested from experiments with B lineage chimeras (3). These chimeras were constructed by reconstituting lethally irradiated mice with syngeneic bone marrow (BM) and peritoneal cells (PerC) from an Ig allotype congenic donor. In these recipients, B cells that express the PerC donor Ig allotype are B-1 cells; conventional B cells with the PerC donor Ig allotype are essentially absent when analyzed by flow cytometry (FACS) (3,9,10). Since many IgA plasma cells are found with the PerC-donor allotype in the gut of these animals up to 1 year after reconstitution, these IgA plasma cells likely belong to the B-1 cell lineage (3). Thus, in addition to PP-derived conventional B cells, B-1 cells contribute significantly to the mucosal IgA response. In studies presented here we provide additional evidence for a role of B-1 cells in generating IgA plasma cells in the gut lamina propria using a totally distinct approach, i.e. the analysis of mice carrying an IgM encoding transgene.

There are a large number of Ig transgenic mouse strains (20 – 29), many of which show B cell developmental defects that alter the relative frequencies of B-1 and conventional B cells, and sometimes block rearrangement of endogenous Ig. B-1 cells in some strains have been shown to co-express endogenous and transgenic Ig or to express mostly endogenous Ig (30 – 32). Conventional B cells in some animals, which may be drastically few in number, express only the transgenic Ig. In other strains the transgene appears to be expressed exclusively in B-1 cells (28). Introduction of transgenes with anti-self specificity can result in the deletion (24,33), anergy (23,27,34), or localization (29) of B cells which express the specific transgene. The aberrations in Ig production reflect the selectability of the transgenic and endogenous Ig molecules expressed by individual B cells, and are influenced by the self-replenishing capability of the B-1 cells that are selected into the peripheral pool.

Previous studies with Sp6 transgenic mice, which carry an Ig μ heavy chain and κ light chain transgene specific for TNP (21) also have demonstrated developmental defects in the B cell population. These studies (21,25,35,36) have collectively shown the following. (i) The number of peripheral B cells in these mice is reduced. (ii) Most B cells express transgenic IgM and a minority of the B cells in peripheral lymphoid organs co-express endogenous and transgenic IgM on their surface. (iii) Despite the low frequency of endogenous IgM bearing cells in these mice, the majority of plasma cells in the spleen contain endogenous Ig heavy chains. Here we extend these findings and demonstrate that the expression of endogenous IgM is mostly restricted to the B-1 cell lineage. Furthermore, we show that these mice contain many IgA plasma cells in the gut lamina propria with or without co-expression of transgenic IgM. The majority of the IgA cells do not express the transgenic idiotype (Id). Thus, most IgA producing cells are the result of an isotype switch from endogenous IgM expressing B cells and largely belong to the B-1 cell lineage. This supports our previous hypothesis that both conventional B cells in the PP and B-1 cells from the peritoneum generate IgA plasma cells in the gut.

Methods

Mice

B6-Sp6 transgenic mice were kindly provided by Dr M. C. Lamers (Max Planck Institute for Immunobiology, Freiburg, Germany). This mouse line is derived from a transgenic mouse carrying a completely rearranged TNP specific μ,κ transgene of BALB/c origin (Igh-C^a allotype) (21). Original Sp6 transgenic mice (the transgene was initially introduced into a Swiss albino mouse) were backcrossed to C57BL/6 (Igh-C^b allotype) mice to facilitate detection of the transgenic IgM (Igh-6a). B6.C20 mice, originally obtained from Dr L. A. Herzenberg (Stanford University Medical School, Stanford, CA) have the Igh-C^a Ig haplotype on a C57BL/6 background. B6-Sp6 transgenic mice and B6.C20 were bred and kept under conventional conditions in the animal facility of the Department of Histology and Cell Biology, University of Groningen. Transgenic mice were bred by mating male B6-Sp6 transgenic mice with female C57BL mice. Mice were tested for expression of the transgene by ELISA. ELISA plates, coated with DNP – BSA, were incubated with serum from mice. Binding of transgene encoded antibody was revealed using an Id specific mouse mAb specific for the transgene (20-5) (kindly provided by Dr M. C. Lamers), following by goat anti-mouse IgG1 conjugated to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). B6-Sp6 mice of both sexes were used at 4 – 11 months of age.

mAbs

The following mAbs were used in this study: rat anti-mouse IgM (331.12) (37), rat anti-mouse IgD (11-26, the generous gift of F. Finkelman), rat anti-mouse B220 (RA3-6B2) (38), rat anti-mouse CD5 (Ly-1, 53-7.3) (39), rat anti-mouse CD11b (Mac-1, M1/70) (40), rat anti-mouse IgA (71.14) (41), mouse anti-mouse Igh-6a (IgM^a, DS-1) (42), mouse anti-mouse Igh-6b (IgM^b, AF6-78.25), mouse anti-mouse Igh-5a (IgD^a, AMS9.1), mouse anti-mouse Igh-5b (IgD^b, AF6-122) (43), mouse anti-mouse Igh-2a (IgA^a, HY16, hybridoma generously provided by M. Potter) (41) and 20-5, a mAb specific for the Id of the transgene (25). For FACS analysis these antibodies were purified and conjugated to FITC, biotin or allophycocyanin (APC) as described previously (44).

Isolation of lamina propria cells

Single cell suspensions from the lamina propria of the small intestine were prepared according to a previously described method (45). Briefly, PP were removed from the small intestine and after rinsing in PBS small fragments (3 cm) were incubated for 15 min at 37°C in Hanks' balanced salt solution (Gibco, Paisley, UK) without Ca and Mg, and supplemented with 1 mM DTT and 5 mM EDTA, to remove epithelial cells. After washing, the fragments were incubated for 2 h at 37°C in 25 ml RPMI 1640 supplemented with 25 mM HEPES, 5% FCS (Gibco), penicillin and streptomycin, containing 0.1 U/ml collagenase (Serva, Heidelberg, Germany) and 200 U/ml DNase I, grade II (Boehringer Mannheim, Germany). The remaining debris was dispersed through a stainless steel strainer and passed over nylon gauze. After washing in RPMI/FCS/HEPES, lamina propria cells were purified using a discontinuous Percoll gradient consisting of a layer of 100% isotonic Percoll (Pharmacia, Uppsala, Sweden) and a layer of 30% Percoll. Cells were harvested from the interface between the 100 and 30% Percoll, washed, and resuspended in RPMI/FCS/HEPES.

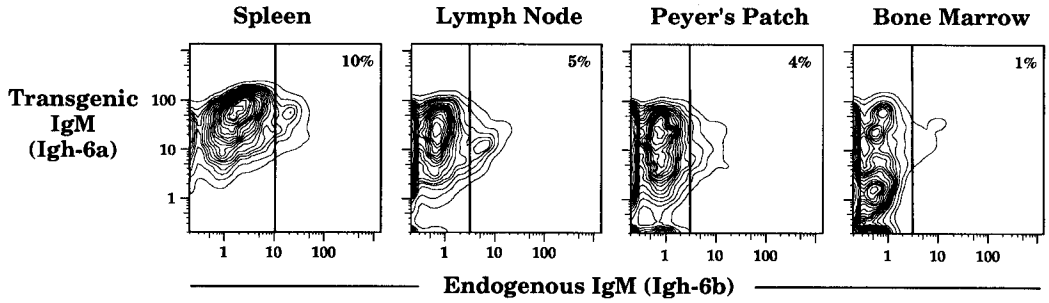

Fig. 1. Most B cells in B6-Sp6 mice express the transgene. Cells from the indicated tissues were stained with anti-B220 (RA3-6B2 either APC conjugated or biotin conjugated; all biotinylated antibodies are revealed with Texas Red – avidin), anti-Igh-6b (AF6-78, fluorescein or APC labeled), and anti-Igh-6a (DS-1, fluorescein or biotin/Texas Red – avidin). Only $B220^+$ lymphocytes are shown. Percentages are given relative to $B220^+$ lymphocytes. In all figures, lymphocytes are shown after gating on forward and obtuse scatter, and dead cells are excluded by appropriate staining and gating with propidium iodide. All plots have 5% probability contours.

Multiparameter flow cytometry

For FACS analysis at Groningen the staining medium was Dulbecco A + B, containing 5% newborn calf serum (Gibco) and 0.1% sodium azide. For FACS analysis carried out at Stanford, the staining medium was deficient RPMI 1640, 10 mM HEPES, pH 7.2, with 3% newborn calf serum and 0.1% sodium azide. Five hundred thousand cells were stained in microtitre plates on ice with a combination of fluorescein, biotin, and APC-conjugated antibodies as described previously (44). Saturating concentrations of antibodies were predetermined and selected to give optimal signal to noise. Biotinylated antibodies were detected with phycoerythrin – streptavidin (Southern Biotechnology Associates) or Texas Red – avidin (44). FACS analysis was performed in Groningen on a FACS 440 (Becton-Dickinson, Mountain View, CA), equipped with argon and HeNe lasers, collecting individual data of 10,000 – 20,000 cells per sample and using Lysis software. Dead cells and debris were excluded from the analysis using forward and sideward profiles. FACS analyses at Stanford were conducted on Flasher, an extensively modified dual laser FACS II, as described (44). Dead cells were stained with propidium iodide and were excluded from the analysis. For each analysis data from 30,000 viable cells were collected and analyzed with FACS/DESK software.

Immunohistological staining procedures

Cytospins were prepared from single cell suspensions of spleen and gut lamina propria by centrifuging 100,000 cells onto microscope slides precoated with 3% BSA. Cryostat sections (7 μm) were made from small gut fragments, snap frozen with liquid freon. Cytospot and frozen sections were fixed for 15 min with ethanol:acetate (50:50, v/v) at – 20°C. For double immunofluorescence staining cytospots/tissue sections were incubated (60 min) with a mixture of FITC-labeled antibodies and biotinylated antibodies or mAb 20-5 (mouse IgG1 anti-transgenic Id) tissue culture supernatants. The biotin was revealed with rhodamine – streptavidin (Jackson Laboratories, West Grove, PA) and mAb 20-5 with rhodamine conjugated to goat anti-mouse IgG1 (Southern Biotechnology Associates). For cytospots, the relative proportion of cells with intense cytoplasmic staining with either reagent or both (i.e. antibody containing cells; plasmablasts and plasma cells) was determined by analyzing at least 300 positive cells with a fluorescence microscope equipped with the appropriate filter sets (Leitz, Wetzlar, Germany).

Table 1. Expression of endogenous and transgenic IgM on B6-Sp6 B cells

B cell population	Tissue				
	Spleen	LN	PP	BM	PerC
Number of animals	(n = 5)	(n = 3)	(n = 2)	(n = 2)	(n = 5)
$B220^+IgM^+$	47 ± 8[a]	29 ± 7	48, 37	6, 6	59 ± 9
$B220^+$Igh-6a$^+$	46 ± 8	28 ± 6	48, 37	6, 5	51 ± 7
$B220^+$Igh-6b$^+$	5 ± 1	2 ± 1	3, 4	<1, <1	25 ± 7
Number of animals	(n = 9)				(n = 13)
Igh-6a$^+$Igh-6b$^-$	48 ± 10	ND	ND	ND	39 ± 9
Igh-6a$^+$Igh-6b$^+$	4 ± 1	ND	ND	ND	19 ± 6
Igh-6a$^-$Igh-6b$^+$	<1	ND	ND	ND	9 ± 7

Cells were stained with fluoresceinated anti-B220, in combination with biotinylated anti-IgM (331), anti-Igh-6a (transgenic IgM), or anti-Igh-6b (endogenous IgM) or with fluoresceinated anti-Igh-6a and biotinylated anti-Igh-6b antibodies. Biotinylated antibodies were revealed with avidin – phycoerythrin or Texas Red – avidin. Additional mice, analyzed with anti-Igh-6a and Igh-6b only give consistent results.

[a]All data represent the percentage (±SD) of total cells with lymphoid forward and sideward/obtuse scatter profiles.

Reconstitution experiments

B6.C20 mice were lethally irradiated (8.5 Gy) and reconstituted 1 day later with i.v. injection of 4 × 10^6 BM cells from B6-Sp6 mice. Seven months after transfer PerC, spleen, BM, LN, and PP were analyzed by FACS.

Results

The majority of B cells in BM and peripheal lymphoid organs of B6-Sp6 transgenic mice express transgenic IgM

FACS analysis confirms and extends previous findings (35,36) that nearly all B cells (defined as $B220^+$ cells) in spleen of B6-Sp6 mice express transgenic IgM and a small proportion expresses endogenous IgM (Fig. 1 and Table 1). Transgenic and endogenous IgM can be distinguished from each other by their allotype: the BALB/c derived transgenic μ heavy chains have the Igh-C^a allotype, while the endogenous μ heavy chains of the C57BL mice carry the Igh-C^b allotype. This allotype difference allows direct and simultaneous detection of transgenic (Igh-6a) and

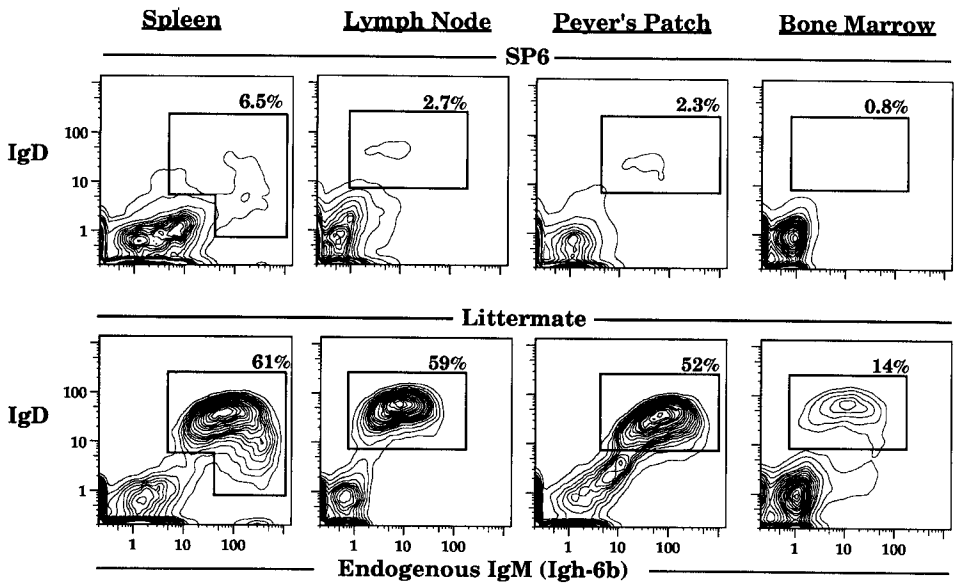

Fig. 2. Endogenous IgM, IgD expressing cells are rare in most tissues of B6-Sp6 mice. Cells from the indicated tissues were stained with anti-IgD (biotin – AF-6-122 or 11-26), anti-Igh-6b (AF6-78, fluorescein or APC labeled), and anti-Igh-6a (DS-1, fluorescein or APC, not shown). Percentages are based on total live lymphocytes.

endogenous (Igh-6b) IgM on individual cells using allotype-specific mAbs. In spleen, ~10% of B cells express endogenous IgM. These data are in close agreement with the results of Grandieu *et al.* (35), but differ from those of Forni (36), who was limited to single-color FACS and concluded that there are higher levels of endogenous IgM expressing B cells. Simultaneous detection of transgenic and endogenous IgM on the cell surface shows that the vast majority of endogenous IgM$^+$ B cells co-express transgenic IgM (Fig. 1 and Table 1). Double-expressing B cells could not be detected in spleens from littermate control animals, B6.C20 mice (Igh-C^a) or (C57BL × BALB/c)F$_1$ hybrid mice (Igh-C$^{a/b}$) (data not shown).

There is also a small fraction of endogenous IgM$^+$ B cells in LN and PP of B6-Sp6 mice (Fig. 1 and Table 1); however, the endogenous IgM$^+$ B cells are rare in the BM. Almost all of the endogenous IgM expressing B cells in LN, PP, and BM co-express transgenic IgM (Fig. 1).

There is some difficulty resolving the endogenous IgM expressing B cells with the staining combination in Fig. 1. These B cells can best be identified by simultaneous staining with allotype specific IgM and IgD, since the IgD staining helps resolve the low IgM staining B cells. The IgD, which is encoded endogenously, is never expressed without endogenous IgM. The endogenous IgM – IgD expressing B cells are compared with a littermate control in Fig. 2. Endogenous Ig expressing (b allotype) cells are present at <5% of normal B cell levels in LN, PP, and BM, and ≤10% of normal levels in spleen.

B-1 cells express endogenous IgM in B6-Sp6 transgenic mice

B-1 cells are distinguished, in part, from conventional B cells by their anatomical localization: B-1 cells predominate in the peritoneal and pleural cavities, are less common in the spleen, and are rare in LN, PP, and BM (5,46). This same localization pattern is observed for the large majority of endogenous Ig expressing B cells in B6-Sp6 mice. Multiparameter FACS analysis

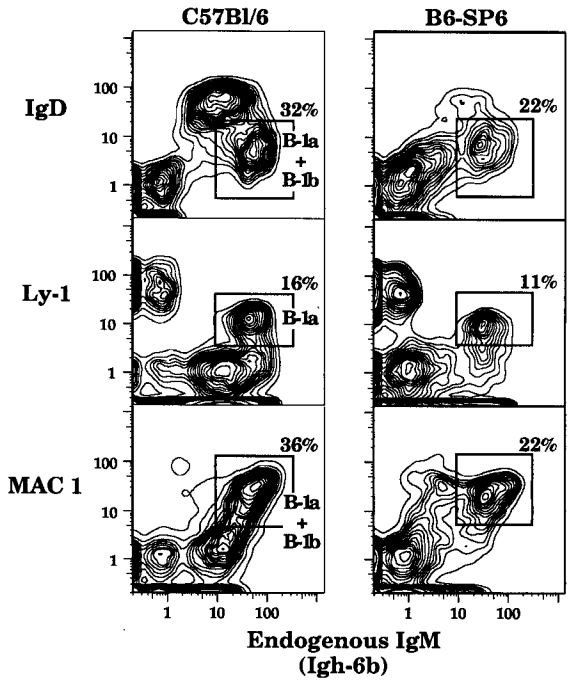

Fig. 3. Endogenous IgM-expressing B cells in the peritoneal cavity of B6-Sp6 mice are B-1 cells. PerC cells from both mice were stained with three reagent combinations: fluorescein – anti-Igh-6b, and (1) biotin – anti-IgD and APC – anti-Igh-6a, (2) APC – anti-Ly-1 and biotin – anti-Igh-6a, or (3) APC – Mac-1 and biotin – anti-Igh-6a. Percentages are based on total live lymphocytes.

of PerC from B6-Sp6 mice shows enrichment of endogenous IgM cells relative to other anatomical locations. More than 40% of the peritoneal B cells express high levels of endogenous IgM (Table 1 and Fig. 3). The Igh-6b$^+$ B-1 cells are present in

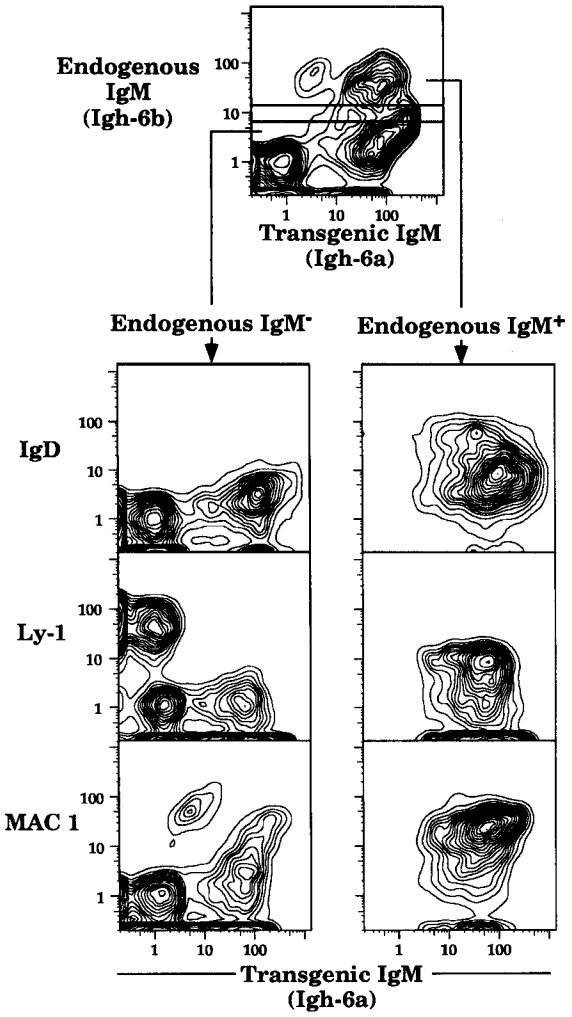

Fig. 4. Most endogenous IgM-expressing B-1 cells in the peritoneal cavity of B6-Sp6 mice also express the transgene. Three-color analysis of B6-Sp6 PerC is presented using the three staining combinations described in Fig. 3. Cells are gated for endogenous IgM expression as indicated in the top panel. Tighter gating on the Igh-6b$^-$ cells does not substantially alter the expression profile of IgD, Ly-1, or Mac-1, and Igh-6a.

the peritoneum of B6-Sp6 at half to equal numbers compared with C57BL littermates.

The vast majority of the endogenous IgM expressing peritoneal B cells have the surface phenotype of B-1 cells. A comparison of a B6-Sp6 transgenic mouse and a littermate is shown in Fig. 3. Almost all peritoneal Igh-6b$^+$ cells express Mac-1 (CD11b) and low levels of Igh-5b (IgD b allotype) as is characteristic for B-1 cells. About 50% of these cells express CD5 (Ly-1) (Fig. 3). Thus, as seen in the littermate, ~50% of the peritoneal B-1 cells belong to the B-1a (CD5$^+$) sublineage and ~50% belong to the B-1b sublineage (which lack CD5 expression and were formerly called 'Ly-1 B sister' cells). A very small fraction of the peritoneal Igh-6b$^+$ B cells have higher levels of IgD and lower levels of IgM, indicating that they may be conventional B cells. These IgD^{br} cells are present at <5% of normal levels in the peripheral lymphoid organs.

Three-color FACS analysis of peritoneal B cells demonstrates

Table 2. Expression of IgM in B6-Sp6 BM recipients

B cell population	Tissue				
	Spleen	LN	PP	BM	PerC
Number of animals	(*n* = 3)	(*n* = 2)	(*n* = 2)	(*n* = 2)	(*n* = 3)
B220$^+$IgM$^+$	37 ± 3[a]	13, 13	50, 53	4, 9	54 ± 21
B220$^+$Igh-6a$^+$	37 ± 3	13, 12	50, 53	4, 9	52 ± 21
B220$^+$Igh-6b$^+$	3 ± 1	<1, <1	2, 2	<1, <1	6 ± 2
Number of animals	(*n* = 3)				(*n* = 3)
Igh-6a$^+$Igh-6b$^-$	37 ± 7	ND	ND	ND	48 ± 20
Igh-6a$^+$Igh-6b$^+$	3 ± 1	ND	ND	ND	4 ± 1
Igh-6a$^-$Igh-6b$^+$	<1	ND	ND	ND	2 ± 1

Cells from lethally irradiated B6.C20 mice reconstituted with B6-Sp6 BM were analyzed 7 months after transfer. Cells were stained with fluoresceinated anti-B220 antibody in combination with biotinylated anti-IgM (331), anti-Igh-6a (transgenic IgM), or anti-Igh-6b (endogenous IgM) or with fluoresceinated anti-Igh-6a and biotinylated anti-Igh-6b antibodies. Biotinylated antibodies were revealed by incubation with Texas Red – avidin.

[a]All data represent the percentage (±SD) of total cells with lymphoid forward and sideward/obtuse scatter profiles.

that the majority (65%) of the endogenous IgM bearing cells, co-express transgenic IgM (Table 1 and Fig. 4). There is a broad range of dual IgM expressing cells in the PerC; 46 – 86% of the Igh-6b$^+$ cells co-express the transgene. Of the tissues analyzed, significant numbers of B cells which express endogenous IgM exclusively are found only in the peritoneum. Gating on the Igh-6b$^+$ cells further demonstrates the co-expression of Igh-6a. The Igh-6b$^+$, Igh-6a$^+$ cells (and relatively few Igh-6b$^+$ only cells) express Mac-1 and low levels of IgD. In contrast, peritoneal B cells that are Igh-6b$^-$ and Igh-6a$^+$, do not express Ly-1 or IgD; however, some are Mac-1$^+$ and may be B-1b cells.

It is difficult to evaluate B-1 cells in spleen and other lymphoid organs since there are no distinct markers available that define all B-1 cells, including B-1b cells. For example, in spleen B-1 cells cannot be defined on the basis of expression of Mac-1 or low expression of IgD and CD23, since B-1 cells in spleen are Mac-1$^-$ and marginal zone B cells also have low levels of IgD and CD23 (32,47,48). The majority of the Igh-6b$^+$ B cells in the B6-Sp6 spleen, which are IgM bright and IgD dull, could be B-1 cells, or perhaps marginal zone B cells (Fig. 2). The rare Igh-6b$^+$ B cells, which express IgD, in the LN and PP appear to be conventional B cells. These cells account for <5% of normal levels seen in the littermate (Fig. 2).

BM from B6-Sp6 mice produces few B-1a cells after transfer into irradiated recipients

Normal adult BM reconstitutes conventional B cells completely, B-1a cells poorly, and B-1b cells moderately well when transferred into lethally irradiated recipients (9,13,49). In B6-Sp6 BM transfers, B cells which only express transgenic IgM are reconstituted with the same success as conventional B cells from normal BM and endogenous Ig expressing B cells are reconstituted with the same pattern as B-1 cells from normal BM. Transfers into lethally irradiated B6.C20 mice (a allotype) yields many Igh-6a$^+$ B cells in recipient spleen, BM, PP, LN, and PerC (Table 2 and Figs 5 and 6). These transgenic IgM$^+$ cells do not co-express Igh-5a (IgD, a allotype) indicating that they are derived from the B6-Sp6

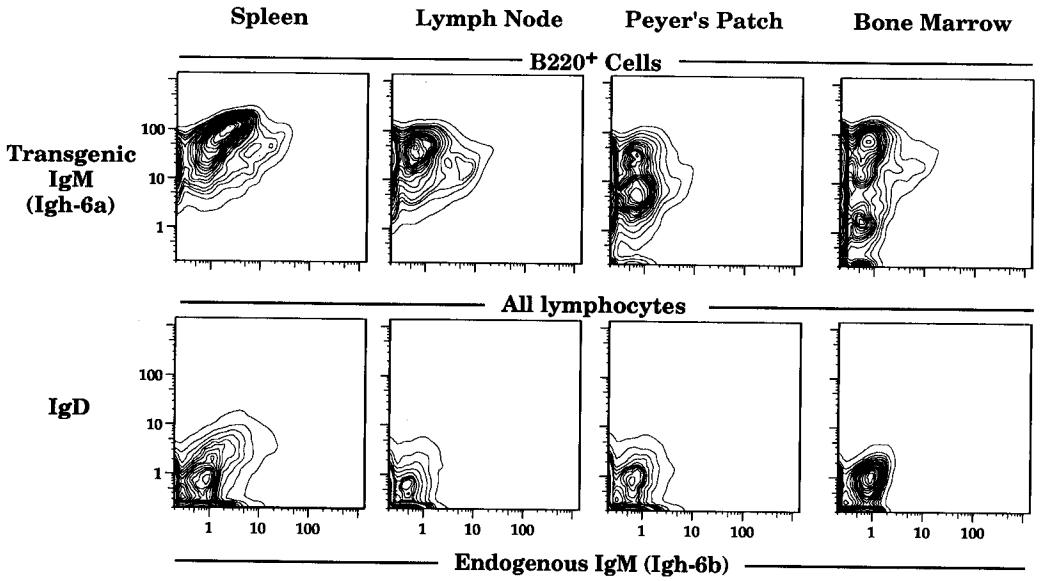

Fig. 5. B6-Sp6 BM reconstitutes endogenous IgM-expressing cells poorly. The same stains described in Fig. 4 are used.

BM and are not host B cells that survived the irradiation. Furthermore, tissue section staining from recipients spleens show virtually all cells in the lymphoid follicles and marginal zones (i.e. B cell areas) express the Id of the transgene (20-5) (data not shown).

Since the recipient is a allotype, B cells expressing Igh-6b can only be derived from the transferred B6-Sp6 BM. A very low frequency of these Igh-6b$^+$ cells is found in recipient spleen, LN, PP, and BM (Table 2 and Fig. 5). Igh-6b$^+$ B cells are more readily detected in the peritoneum (Fig. 6), but their numbers are still low (Table 2). Most of these cells are also positive for the transgene (Table 2). The BM-derived Igh-6b$^+$ B cells are phenotypically B-1b cells: they are dull for IgD, positive for Mac-1, and lack surface CD5 (Fig. 6). Like normal mice (9,13,49), B6-Sp6 BM repopulates B-1b cells better than B-1a cells. Thus, by the criteria of anatomical localization, cell surface phenotype, and reconstitution pattern from BM, the vast majority of endogenous Ig expressing B cells in B6-Sp6 mice are B-1 cells.

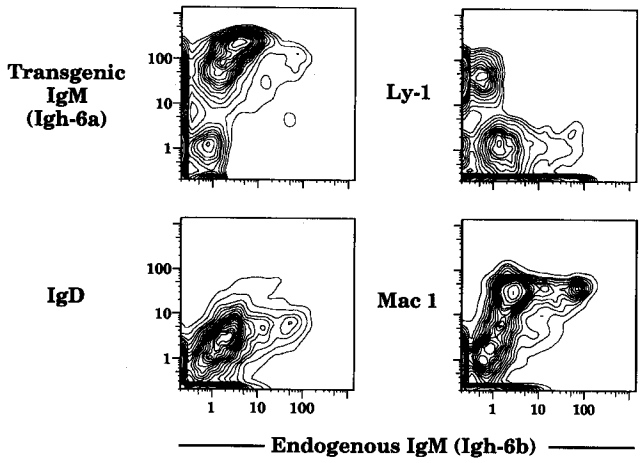

Fig. 6. B6-Sp6 BM reconstitutes endogenous IgM-expressing peritoneal B-1 cells poorly. The staining combinations are described in Figs 1 and 2.

Many IgA containing cells are found in the intestinal lamina propria of B6-Sp6 mice

Immunofluorescence staining of sections from the gut with monoclonal rat anti-mouse IgA shows high numbers of IgA containing cells in the lamina propria (Fig. 7). In transgenic mice, but not control mice (littermates, B6.C20, and BALB/c mice), high numbers of IgM containing plasma cells are also found in the gut. Staining with allotype specific reagents demonstrates that this IgM is exclusively of transgenic origin. Two-color staining of cytospins from isolated lamina propria cells for IgA and IgM reveals that the vast majority of the IgM containing cells in the lamina propria, which are about one-third of the plasma cells/blasts, also have cytoplasmic IgA. Very few cells (2%) contain only transgenic IgM (Table 3).

In spleen, ~20% of the IgM containing cells express endogenous IgM (Table 3). Half of these cytoplasmic Igh-6b$^+$ cells (i.e. 10% of all IgM containing cells) also contain transgenic IgM. The remaining 80% of the splenic IgM plasmablasts and plasma cells contain only transgenic IgM [but may co-express other endogenous heavy chains as shown by Forni (36)]. The splenic plasmablast/cell frequencies reported here are consistent with data presented by others (35,36).

The IgA in the B6-Sp6 gut is encoded by endogenous α heavy chains, since only a μ heavy chain transgene was introduced into these mice. Furthermore, staining of tissue sections from transgenic guts with IgA or IgM in combination with an antibody directed to the Id of the transgene (20-5) shows that all IgM containing cells and only a minor proportion of IgA containing cells also react with the anti-Id mAb (Fig. 7). These Id$^+$ IgA cells likely represent IgA plasmablasts/cells that also contain transgenic IgM. Thus, these IgA containing cells in the gut lamina propria cells of B6-Sp6 mice are most likely the result of an isotype switch

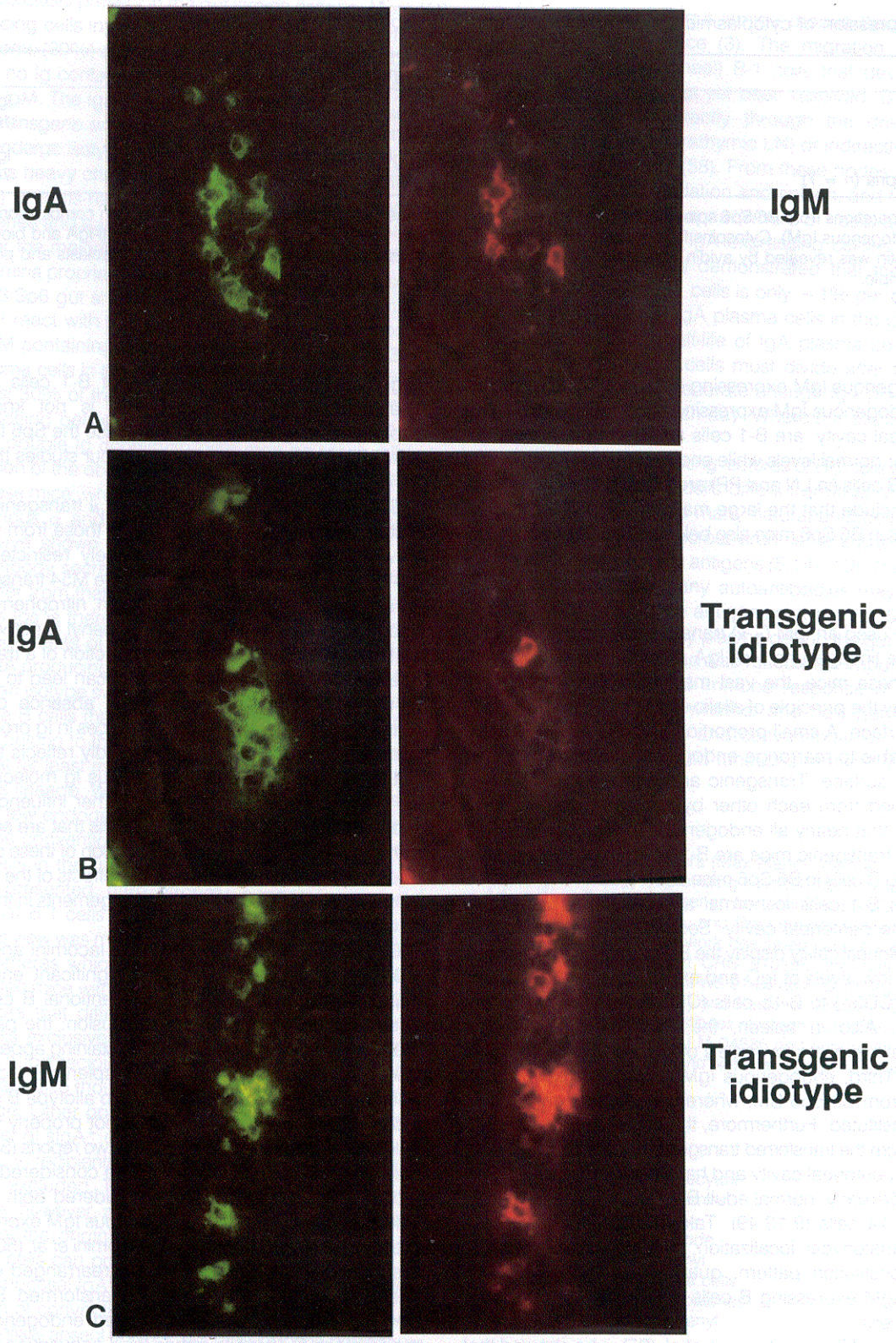

Fig. 7. The antibody containing cells in the gut lamina propria of B6-Sp6 mice are of two main types: endogenous IgA only and endogenous IgA plus transgenic IgM. Frozen sections of B6-Sp6 guts were double stained with: (a) fluoresceinated anti-IgA (mAb 71.14) and biotinylated anti-IgM (mAb 331); (b) fluoresceinated anti-IgA in combination with mAb 20-5 (mouse anti-transgenic Id); (c) fluoresceinated anti-IgM and mAb 20-5. Biotinylated antibodies were revealed by avidin conjugated to rhodamine and the 20-5 mAb by a goat anti-mouse IgG1 conjugated to rhodamine (to detect 20-5 antibody). Control antibodies included irrelevant fluoresceinated mouse anti-rat and anti-mouse antibodies, and these reagents did not give any staining (not shown).

Table 3. Expression of cytoplasmic IgM in spleen and gut of B6-Sp6 mice

	Proportion antibody containing cells		
	Igh-6a$^+$Igh-6b$^-$	Igh-6a$^+$Igh-6b$^+$	Igh-6a$^-$Igh-6b$^+$
Spleen (n = 3)	80 ± 12[a]	10 ± 6	10 ± 7
	IgM	IgM + IgA	IgA
Gut lamina propria (n = 7)	2 ± 2	28 ± 8	70 ± 7

Cytospin preparations from B6-Sp6 spleen were double stained with fluoresceinated anti-Igh-6a (transgenic IgM) in combination with biotinylated anti-Igh-6b (endogenous IgM). Cytospins from isolated gut lamina propria cells were stained with fluoresceinated anti-IgA and biotinylated anti-IgM (mAb 331). Biotin was revealed by avidin conjugated to rhodamine. At least 300 antibody containing cells (plasmablasts and plasma cells) were scored per sample.

[a]Mean ± SD.

from an endogenous IgM expressing cell to IgA. Since the vast majority of endogenous IgM expressing B cells, especially those in the peritoneal cavity, are B-1 cells and since these cells are present at near normal levels while endogenous IgM expressing conventional B cells (in LN and PP) are present at <5% normal levels, we conclude that the large majority of the intestinal IgA containing cells in B6-Sp6 mice also belong to the B-1 cell lineage.

Discussion

Here we have used an IgM (μ,κ) transgenic mouse (B6-Sp6) to investigate the lineage origin of IgA plasma cells in the small intestine. In these mice, the vast majority of B cells express, as expected by the principle of allelic exclusion, only transgenic IgM on the surface. A small proportion of the B cells, however, is clearly still able to rearrange endogenous Ig and to express them on their surface. Transgenic and endogenous IgM can be distinguished from each other by their allotype. We have demonstrated that nearly all endogenous IgM expressing cells in B6-Sp6 μ,κ transgenic mice are B-1 cells. First, endogenous IgM expressing B cells in B6-Sp6 mice show the same anatomical localization as B-1 cells in normal animals. They are clearly enriched in the peritoneal cavity. Second, endogenous IgM$^+$ cells in the peritoneal cavity display the B-1 cell surface phenotype. They express low levels of IgD and are positive for Mac-1. The ratio of B-1a (CD5$^+$) to B-1b cells (CD5$^-$) in B6-Sp6 is similar to littermates. Also in spleen, the (few) endogenous IgM expressing cells express low levels of sIgD and may therefore be B-1 cells. Third, endogenous IgM expressing cells do not transfer well from B6-Sp6 BM, whereas transgene-only B cells are fully reconstituted. Furthermore, the few endogenous IgM$^+$ cells derived from the transferred transgenic BM are predominantly located in the peritoneal cavity and have the phenotype of B-1b cells (CD5$^-$). Similarly, normal adult BM reconstitutes B-1b cells better than B-1a cells (9,13,49). Taken together, these three criteria, i.e. anatomical localization, cell surface phenotype, and BM reconstitution pattern, qualify the large majority of endogenous IgM expressing B cells in B6-Sp6 μ,κ transgenic mice as B-1 cells.

These findings differ with Lamers *et al.* (25), who showed that ~33% of the B cells in the spleen express endogenous IgM together with high levels of (endogenous) IgD and therefore might well be conventional B cells. Furthermore, they demonstrated that the antibody response to dextran, usually produced by B-1 cells (17), is significantly impaired in Sp6 transgenic mice, suggesting the relative absence of B-1 cells in these mice. The reason for this discrepancy is not known, but one explanation might be that in their mice the Sp6 transgene is on a BALB/c background, whereas in our studies the transgene is on a C57BL background.

Comparing B6-Sp6 mice to other μ transgenic mice we find that our data are strikingly similar to those from the M54 strain, in which endogenous IgM is largely restricted to B-1 cells (30–32). As with the B6-Sp6 mice the M54 transgene is specific for an exogenous (laboratory) hapten, nitrophenyl. Like the Sp6 encoded specificity for TNP, nitrophenyl is not well recognized by B-1 cells (17). In contrast, introduction of a transgene whose specificity is preferred by B-1 cells can lead to the expression of transgene-only B-1 cells in the absence of endogenous rearrangements (28). These differences in Ig production among transgenic mouse strains undoubtedly reflects the selectability of the transgenic Ig and endogenous Ig molecules expressed by the B cell subsets. They are further influenced by the self-replenishing capability of the B-1 cells that are selected into the peripheral pool. However, the operation of these selective factors does not preclude other differential effects of the transgene, e.g. selective interference with Ig rearrangements in the development of conventional B versus B-1 cells.

Other studies with M54 mice by Iacomini and Imanishi-Kari (50–52) conclude that there is significant endogenous IgM expression on both B-1 and conventional B cells. The FACS analysis does not justify this conclusion: the panel of staining reagents is too limited; background staining appears high; Mac-1 is incorrectly used as a marker for splenic B-1 cells rather than peritoneal B-1 cells; a allotype and b allotype B cells are poorly resolved, and the gates are often not properly set. This group ignored the existence of B-1b cells in two reports (50,51); peritoneal conventional B and B-1b cells were considered as one group. More recently, however, they considered both B-1a and B-1b cells, and concluded that endogenous IgM expressing cells are present in both populations (52). Iacomini *et al.* (50) also observed a small percentage of functionally rearranged endogenous Ig heavy chain genes in Abelson transformed BM pre-B cells from M54 mice. These BM derived endogenous IgM$^+$ cells may be pre B-1b cells rather than conventional B cells. B-1b cells are reconstituted by adult BM from non-transgenic strains (13,49). Furthermore, as we have shown here, endogenous IgM expressing B cells in the peritoneum of B6-Sp6 BM recipients are B-1b cells.

In this paper we have demonstrated that there are many IgA

plasma cells/blasts present in the gut lamina propria. Most of the IgA producing cells in the gut have only IgA in their cytoplasm although some (30%) also contain transgene coded μ. There are essentially no Ig containing cells in the gut which only express transgenic IgM. The IgA is produced by endogenous C_α genes, since the transgene encodes only for C_μ. Certain μ transgenic mice can undergo isotype switching between transgenic IgM and endogenous heavy chain genes (53) either by *trans*-splicing of mRNA (54) or *trans*-recombination (55) between endogenous C_α or C_γ genes and transgenic V_H genes. This is not likely to be the case for the majority of the IgA expression of plasma cells in the gut lamina propria. First, two-color staining of tissue sections from the B6-Sp6 gut shows that the majority of IgA containing cells do not react with the 20-5 Id encoded by the transgene, while all IgM containing cells express this Id. Since ~30% of the IgA plasma cells in the intestine also contain (Id^+) transgenic IgM, at most 30% of the IgA plasma cells can be the result of isotype switching between endogenous C_μ and transgenic V_H genes. The double Ig containing cells may be responsible for the production of the observed chimeric IgM/A molecules in the serum of these mice, which are composed of Ig chains encoded by endogenous α and transgenic μ (56). Second, Grandieu *et al.* (57) have shown that the endogenous V_H gene family usage of antibody secreting cells in the spleen of B6-Sp6 mice does not differ from that of normal B6 mice. The expression of endogenous Igs is therefore the result of complete, functional VDJ rearrangements of endogenous heavy chain genes. The majority of IgA producing cells in the intestine are thus most likely the result of an isotype switch from endogenous IgM expressing B cells. Since B cells that express endogenous IgM in B6-Sp6 mice largely belong to the self-replenishing B-1 cell lineage, we conclude that (at least most) intestinal IgA producing cells also belong to this lineage. We cannot totally exclude the possibility that the very few endogenous IgM^+ cells found in LN and PP, which may be conventional B cells, also contribute to the IgA containing cells in the gut.

The data presented here strongly support our previous hypothesis that B-1 cells are active in mucosal IgA responses (3,58,59). This view was mainly based on experiments with stable B lineage chimeras which were constructed by reconstituting lethally irradiated mice with BM and peritoneal cells with congenic pairs of donors that differ in Ig allotype. In these chimeras, peritoneal cells exclusively give rise ot B-1 cells. Since many plasma cells in the recipient gut were from the peritoneal cell donor, these are also thought (but not proven) to belong to the B-1 cell lineage. Other possibilities, however, like expansion of the low number of $sIgA^+$ cells (memory B cells) present among the donor PerC in recipients irradiated gut (3), although less likely, cannot yet be excluded. The findings with the transgenic mice described here, however, provide a distinct approach to address the question of the origin of IgA plasma cells in the gut and show that B-1 cells can potentially play a significant role in the generation of mucosal IgA plasma cells. Apparently, in addition to a population of conventional B cells located in PP, B-1 cells, which are essentially absent from PP but present in the peritoneum, participate in mucosal IgA response. The relative contribution of each of these two lineages in the production of mucosal IgA in normal, untreated mice, is not exactly known. However, the studies in chimeric mice suggest that B-1 cells may contribute significantly to the IgA plasma cell pool: up to 40% of the IgA plasma cells in the gut lamina propria belong to the B-1 cell lineage in these mice (3). The migration and differentiation pathways of (peritoneal) B-1 cells that develop to these IgA plasma cells have not yet been resolved. B-1 cells may either leave the cavity directly through the draining LN (caudal medinastinal and parathymic LN) or indirectly by migrating first to the pleural cavity (58). From these nodes B-1 cells probably enter the blood circulation and spleen, and finally they migrate into the gut lamina propria. Where B-1 cells actually switch from IgM bearing cells to IgA expressing cells is not known. BrdU incorporation studies demonstrated that the turnover rate of peritoneal (IgM^+) B-1 cells is only ~1% per day (60,61). Given the high number of IgA plasma cells in the gut in combination with an estimated half-life of IgA plasma cells of 5 days (62), this implies that B-1 cells must divide after they have left the peritoneal cavity to generate enough IgA plasma cells that are needed in the gut each day. Where this expansion takes place is also not known.

One of the most exciting features of B-1 cells is that the antibody repertoire of the B-1 cells differs from that of the conventional B cells. They produce many natural antibodies (which may be directed to common microenvironmental antigens) and antibodies to microorganism coat antigens (5,14 – 19). The observation that B-1 cells produce many autoantibodies may even be due to cross-reacting epitope antigens on bacteria (63). Therefore it is not a surprise that many plasma cells in the gut appear to belong to this B cell lineage and also reflects the importance of B-1 cells in the overall humoral immune response of the animal. The intriguing question of whether and how gut IgA plasma cells derived from B-1 cells and conventional B cells differ in their antibody repertoires and functions remains to be answered.

Acknowledgements

We thank Dr M. C. Lamers (Max Planck Institute for Immunobiology, Freiburg, Germany) for providing us with B6-Sp6 mice. We thank Sharon Adams and Gerrit Jan Deenen for technical assistance, Peter van der Sijde for the photographs, and Drs Leonore A. Herzenberg and Nico Bos for discussions. The work described here is supported in part by NATO collaborative grant CRG 910195, the Netherlands Digestive Diseases Foundation, grant WS 91-25 (to F. G. M. K.), the Interuniversity Institute for Radiopathology and Radiation Protection, I.R.S. grant 7.2.3 (to F. G. M. K.), National Institutes of Health grant HD-01287 and National Library of Medicine grant LM 04836 (to Leonore A. Herzenberg), and DK38707 pilot study 1-04 and National Research Service Award AI07937 (to A. B. K.).

Abbreviations

APC	allophycocyanin
BM	bone marrow
Id	idiotype
LN	lymph node
NP	nitrophenyl
PerC	peritoneal cells
PP	Peyer's patches
TNP	trinitrophenyl

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