RELATIONSHIP BETWEEN EXPRESSION OF IgA BY PEYER'S PATCH CELLS AND FUNCTIONAL IgA MEMORY CELLS

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The formation of a complete Ig γ or α chain gene involves two recombinational events (1–3). The first recombination, V-D-J joining, brings V_H adjacent to $C\mu$. The second recombination, the heavy chain switch, brings the same V_H gene adjacent to a different C_H gene, allowing the expression of the original V_H gene with one of the $C\gamma$ or $C\alpha$ genes. Several analyses of Ig heavy chain gene content and context using a variety of myelomas and hybridomas have shown that secretion of non-IgM isotypes is associated with the deletion of C_H genes 5' to the expressed C_H gene (4–10). These data suggest that once a cell expresses a particular non-IgM isotype its clonal progeny would be restricted to expression of that non-IgM isotype and those isotypes encoded by C_H genes occurring 3' to its own C_H gene at the Igh locus. However, if C_H gene deletion occurs as a consequence of sister chromatid exchange, as several studies have suggested (11–13), it would be possible for clonal progeny to express a C_H gene originally 5' to that expressed by their progenitors. Regardless, the cell lines studied represent secretory plasmablasts at the terminal stages of B lymphocyte maturation, and C_H gene deletion may not necessarily be the only mechanism for B cell differentiation with respect to isotype expression. For example, it has not been clearly established that B cells expressing non-IgM isotypes as membrane Ig have undergone any deletions of C_H genes. In fact, recent studies with both splenic B lymphocytes that express dual membrane isotypes, $IgM^+/IgG1^+$ or IgM^+/IgA^+, and a B cell lymphoma that expresses and secretes both IgM and IgG1, have been interpreted as indicating that expression of non-IgM isotypes in earlier stages of differentiation may not be accompanied by C_H gene deletion (14, 15). Furthermore, the majority of antigen-specific clones derived from enriched antigen-primed $sIgG^+$ splenic B cells secreted IgM, suggesting that the expression of non-IgM isotypes on memory B cells does not reflect a restriction in isotype potential (16).

It has been established that the Peyer's patches (PP)[1], compared with other lymphoid tissues, are an enriched source of precursors of IgA-secreting cells (17), and these appear to lack surface IgM (sIgM) and bear allotypic markers associated with the α chain of IgA (18, 19). However, the surface phenotype of functional IgA memory cells in the PP has not been determined. These memory cells have been operationally defined as being able to respond to stimulation by a thymus-dependent antigen to give clones of plasma cells, all of which secrete only IgA (20). One interesting and unresolved question is the relationship between sIgA-bearing B cells and IgA memory cells. Consequently, the relationship between sIgA expression on PP cells and isotype display by their clones following in vitro antigenic challenge was investigated. Furthermore, since it has been inferred that germinal centers are the site at which both memory cells and plasmablasts are generated (21–23), the relationship between peanut agglutinin (PNA) binding, a characteristic of germinal center cells (24), and clonal isotype display upon in vitro antigenic challenge was also investigated. This study demonstrated that, in general, the IgA memory B cell population in murine PP consists of small, resting sIgA$^+$, PNA^{low}, sκ^{high} cells that make relatively low levels of IgA-specific mRNA. Furthermore, there appears to be a minor population of sIgA$^-$ cells that gives rise to clones secreting IgA exclusively, the operational definition of IgA commitment, suggesting that restriction to IgA secretion may occur without detectable sIgA expression. Finally, although the PNA^{high} germinal center cells account for the majority of sIgA$^+$ PP cells, they do not generate IgA-secreting clones in splenic fragment cultures in response to antigen added at the initiation of culture.

Materials and Methods

Animals.   BALB/cByJ mice were bred and maintained in our animal facility at the University of Pennsylvania. Mice were used between 8 and 12 wk of age.

Preparation of Reagents for Immunofluorescence.   Rabbit anti-mouse IgA was prepared from the serum of New Zealand white rabbits immunized with either myeloma protein MCPC 603 or the Fab fragment of myeloma protein MOPC 167. The globulin fractions were absorbed on affinity columns of normal mouse IgG coupled to Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) and myeloma protein CBPC 112 (IgM)-Sepharose, and then isolated on an affinity column of myeloma protein CBPC 4 (IgA)-Sepharose. The two anti-IgA preparations were pooled and separate portions were labeled with ^{125}I or biotinylated. The ^{125}I-labeled anti-IgA was tested for isotype specificity by solid-phase RIA as described (25) and failed to react with a panel of antiphosphocholine mAbs, including IgM, IgG1, IgG2, and IgG3 isotypes (25), while scoring the monoclonal IgA, S107, over a range of 0.25–20 ng. The isotype specificity of the biotinylated anti-IgA was further tested using the following monoclonal cell lines, fluorescein-labeled avidin, and fluorocytometry: (*a*) a tissue-culture adapted line of MOPC 315, which bears sIgA (gift of Richard Hoover, University of Pennsylvania); (*b*) X16c8.5, which is a B cell lymphoma bearing both sIgM and sIgD (see reference 26); and (*c*) the W231.2.1a line, which bears and secretes IgM (see reference 26). The biotinylated anti-IgA stained only the MOPC315 cells, biotinylated anti-IgD stained only the X16c8.5 cells, and fluorescein-labeled anti-IgM stained X16c8.5 and W231.2.1a cells.

Anti-I-A was mAb BP107.3 (27). Biotin was conjugated to antibodies using a previously described procedure (28). PNA (Boehringer-Mannheim Biochemicals, Indianapolis, IN)

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[1] *Abbreviations used in this paper:*   Hy, hemocyanin; In, inulin; PC, phosphocholine; PNA, peanut agglutinin; PNA$^{high\ or\ low}$, binding high or low levels of PNA, respectively; PP, Peyer's patches; TR, Texas Red.

was conjugated to biotin or Texas Red (TR; Molecular Probes, Inc., Junction City, OR) in the presence of 200 mM galactose (29). Fluorescein (FITC)-conjugated anti-κ prepared from the supernatant of hybridoma 187 (30) was the gift of J. Davie, Washington University, St. Louis, MO.

Preparation and Purification of Lymphocytes.   Single cell suspensions of lymphocytes were prepared from the PP of 6–12 mice and resuspended at a concentration of 8×10^7 cells/ml in PBS supplemented with 5% FCS and 0.05% sodium azide. In some experiments PP were depleted of T cells by treatment with mAb 30H12 (31), anti-Thy-1, and complement. The resulting population was 90% sκ^+, as judged by immunofluorescence using a FACS IV (Becton Dickinson & Co., Mountain View, CA). To separate lymphocyte subsets, cell suspensions were incubated for 30 min on ice with a biotin-conjugated reagent, either anti-IgA or PNA, washed three times in PBS, 5% FCS, and 0.05% sodium azide, and resuspended in a volume equal to the original volume of cells of an appropriate dilution of either FITC-avidin or TR-avidin and FITC-anti-κ. After incubation on ice for 30 min, cells were washed three times as described and resuspended at 10^7 cells/ml for sorting on the FACS IV. For analysis on the FACS IV the same procedure was followed with 20 μl of cells at 8×10^7 cells/ml.

Cell Cycle Analysis.   For cell cycle analysis of PP subpopulations cells were first stained with biotinylated antibodies and FITC-avidin as described. After the last wash, cells were pelletted, resuspended in 200 μl of cold 70% ethanol, and incubated for 10 min on ice. The cell suspensions were pelleted and resuspended in 100 μl RNAase A (Sigma Chemical Co., St. Louis, MO) at a concentration of 62 μg/ml in PBS and incubated for 30 min at 37°C. After incubation with RNAase, 100 μl of propidium iodide (Sigma Chemical Co.) at 0.36 μg/ml in PBS was added to each well. Cell cycle analysis was performed using a Cytofluorgraf 50H connected to a Data General MP/200 microprocessor (Ortho Diagnostics Systems Inc., Westwood, MA) as described (32).

Antigens.   Phosphocholine (PC), inulin (In), and trinitrophenyl (TNP) were coupled to *Limulus* hemocyanin (Hy) as previously described (20). Preparations with coupling ratios of 10, 30, and 22 mol, respectively, per 100,000 g Hy were used.

Enumeration of Precursor Cells.   Splenic fragment assays were performed as described (33). Briefly, either whole or fractionated PP cell suspensions were injected into lethally irradiated (1,650 rad) Hy-primed (100 μg in CFA [Difco Laboratories Inc., Detroit, MI] injected intraperitoneally at least 5 wk before use) recipients. Cultures were stimulated with 5–10 μg/ml of hapten-carrier conjugates. Supernatants were screened and isotyped by solid-phase RIA using reagents whose preparation and specificity have previously been described (25). Productive clones are operationally and consistently defined as splenic fragment cultures yielding supernatants that contain detectable antibody on at least two consecutive days of harvest spaced 3 d apart and that can subsequently be successfully isotyped. Data are presented from experiments yielding $\leq$10% positive fragment cultures.

Cytoplasmic Dot Blots.   Cytoplasmic dot blotting was performed essentially as described (34), with the modification that cells were lysed in 10 mM Tris, pH 7.0, 1 mM EDTA, and 1,000 U/ml human placental RNAase inhibitor (Bethesda Research Laboratories, Bethesda, MD). Dot blots were prehybridized for 24 h at 45°C in 50% formamide, 5× Denhardt's reagent (1× = 0.02% wt/vol Ficoll, 0.02% wt/vol polyvinylpyrrolidone, and 0.02% wt/vol BSA Pentax Fraction V [Sigma Chemical Co.] in distilled water), 5× SSC (1× = 0.15 M NaCl, 0.015 M Na$_3$C$_6$H$_5$O$_7$, pH 7.0), and 200 μg/ml salmon sperm DNA (Sigma Chemical Co.). Blots were hybridized for 72 h at 45°C in 50% formamide, 2× Denhardt's reagent, 0.1% SDS, 5× SSC, 100 μg/ml salmon sperm DNA, and 10^7 cpm of ^{32}P-labeled PαJ558 (35) prepared by nick translation using α-[^{32}P]deoxycytidine triphosphate (Amersham Corp., Arlington Heights, IL). Blots were washed twice for 20 min in 0.1% SDS and 2× SSC and twice for 20 min in 0.1% SDS and 0.2× SSC. All washes were done at room temperature. After washing, blots were autoradiographed for 2–4 d using Kodak X-OMAT AR film and a Cronex intensifying screen (DuPont Co., Wilmington, DE).

Densitometry.   Densitometry was performed using a Zeineh Soft Laser Scanning Densitometer (Biomed Instruments, Inc., Fullerton, CA) interfaced to an Apple II computer.
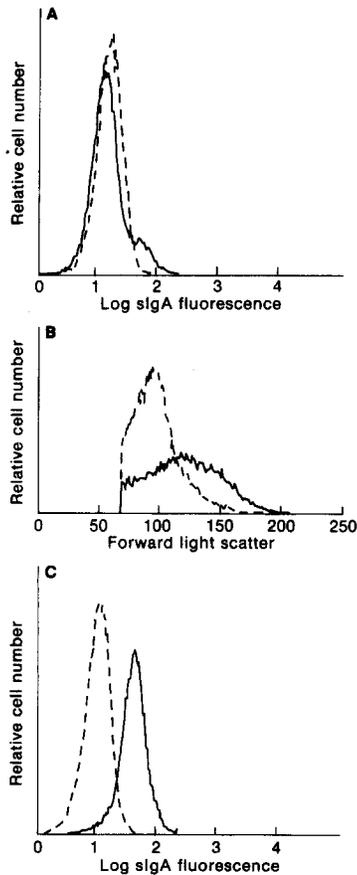
FIGURE 1.   T-depleted PP cells were stained with biotin–anti-IgA and FITC-avidin and separated using a FACS IV. (A) Analysis of total population. (– – – –) FITC-avidin only, (———) biotin–anti-IgA and FITC-avidin. (B) Separated populations analyzed for light scatter. (– – – –) sIgA⁻, (———) sIgA⁺. (C) Separated populations analyzed for fluorescence. (– – – –) sIgA⁻, (———) sIgA⁺.

All scans were made at the same absorbance on the same intensity scale. Curves from scans were integrated and the area under the curve generated by a particular dot is indicative of relative intensity. Comparisons of relative intensity are made at the indicated cell concentration that occurs at a point where differences in intensity are linear within all samples being compared.

Results

sIgA Expression and Isotype Display.   To determine the relationship between sIgA expression and isotype display, PP cells from normal mice were separated into $sIgA^-$ and $sIgA^+$ populations by FACS. Fig. 1 shows the results of sorting of T cell–depleted PP cells on the basis of sIgA staining. Whole PP cell suspensions were ~7% $sIgA^+$. After sorting and reanalysis the $sIgA^+$ population was 53–67% $sIgA^+$ and the $sIgA^-$ population was <1% $sIgA^+$. All of the clones derived from $sIgA^+$ PP cells secreted exclusively IgA (Table I). Thus, by the operational definition of IgA commitment, i.e., precursors that give rise to clones secreting exclusively IgA, $sIgA^+$ cells are committed to IgA. Furthermore, the $sIgA^+$ population was enriched for In-specific precursor cells (6.5 vs. 2.7 per 10^5 B cells in the unfractionated population), whereas the $sIgA^-$ population was depleted of these precursors (9 vs. 27 per 10^6 B cells in the unfractionated

TABLE I

Isotype Display of sIgA⁺ and sIgA⁻ PP B Cells

Isotype expressed	Clones derived from cell types:			
	PP	sIgA⁻	sIgA⁺	sIgA⁺ + sIgA⁻
			%	
IgM only	9	3	0	2
IgA only	63	39	100	57
IgM + IgA	7	16	0	11
IgG + IgA	18	19	0	14
IgM + IgG + IgA	3	23	0	16
Total number of clones	70	31	13	44
Number of In-specific clones	39	13	13	26
In-specific frequency*	27	9	65	17

PP cells were sorted into the indicated populations: sIgA⁻ and sIgA⁺. PP indicates that the clones were derived from PP cells that were not sorted into subpopulations. Cells were injected into lethally irradiated Hy-primed recipients for splenic fragment assay. Cultures were stimulated with In-Hy, TNP-Hy, and PC-Hy. sIgA⁺ + sIgA⁻ is the summation of clones from the sIgA⁺ and sIgA⁻ subpopulations.

* Frequency per 10⁶ B cells is determined using Poisson analysis to determine the number of precursor cells and is calculated on the basis of 4% lodging, whole PP and sIgA⁻ are 60% B cells, and sIgA⁺ are 100% B cells.

population). The sIgA⁻ population accounts for all of the clones derived from fractionated PP cells that secrete isotypes other than IgA. However, it does appear that sIgA⁻ cells also contain a minor population that is committed to IgA since 39% of the clones derived from this population, which is depleted in In-specific precursors, secreted exclusively IgA. These observations suggest that IgA commitment may not necessarily be reflected by sIgA expression as detectable by FACS analysis. Thus, to ascertain if there are members of the sIgA⁻ subpopulation of PP cells that are detectable based on expression of mRNAα that have simply not yet expressed sIgA, cytoplasmic dot blots of the same PP subpopulations were probed using a radiolabeled cDNA probe for mRNAα. The IgA-secreting myeloma MOPC 315 was used as a positive control, and thymocytes were used to establish a background level for probe sorption. In two experiments, of which Fig. 2 is representative, no mRNAα was detectable in sIgA⁻ populations. In contrast, the sIgA⁺ population is substantially enriched in mRNAα (Fig. 2).

Characterization of IgA Memory Cells. Fig. 1B shows that sIgA⁺ cells are on the average larger than other PP B cells based on forward light scattering analysis. These data correlate with the observation that the majority of sIgA⁺ cells are in germinal centers (29). Furthermore approximately half of sIgA⁺ cells are in G_0, G_1 phase of the cell cycle and the other half are in S, G_2, and M (Table II), whereas only 14% of PP B cells, defined by staining for I-A, are in S, G_2, and M. Although sIgA⁺ cells are a small subset of PP, they can be divided into two populations, sκ^{high} and sκ^{low} (Fig. 3). Correspondingly, all PP B cells can also be divided into two distinct subsets (Fig. 4), PNA^{high},sκ^{low} and PNA^{low},sκ^{high}, the former having a surface phenotype characteristic of germinal center B cells (29). Since more than half of the sIgA⁺ cells in the PP appear to be in germinal centers
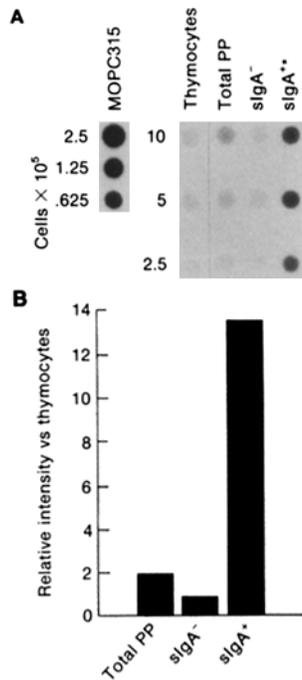
A

B

FIGURE 2.   T-depleted PP cells were stained with biotin–anti-IgA and FITC-avidin and separated into the indicated populations using a FACS IV. (*A*) Cytoplasmic dot blots probed for α-specific mRNA. (*) Serial dilutions of mIgA$^+$ cells started at 5×10^5 cells. (*B*) Densitometry comparing 2.5×10^5 cells from indicated populations with thymocytes.

TABLE II

Cell Cycle Analysis of PP B Cell Subpopulations

Population	Cells*	
	G_0, G_1	S, G_2, M
	%	
I-A$^+$	84.6	14.0
sIgA$^+$	57.4	41.6

PP were removed from normal mice. Immunofluorescence and cell cycle analysis were performed as described in Materials and Methods.
* Percentage of cells from the indicated populations that are in G_0, G_1 or S, G_2, M as determined by propidium iodide binding.

(29, Fig. 3), these PNA^{high},sκ^{low} germinal center B cells were tested for their content of functional IgA memory cells in antigen-dependent splenic fragment cultures in comparison with B cells found in the other PP subpopulation. Using the levels of sκ and PNA binding the two major PP B cell subpopulations, the PNA^{high},sκ^{low} germinal center cells and the PNA^{low},sκ^{high} B cells were separately isolated (Fig. 4*A*). Fig. 4*B* shows a reanalysis of these subpopulations typical of eight consecutive preparative sortings, indicating how well the two subsets were resolved. Table III documents that the PC- and In-specific clonal precursors, including the IgA memory cells and those that give clones expressing some IgA, are found in the majority PNA^{low},sκ^{high} subpopulation. These precursors are mostly antigen-dependent, i.e., they require the presence of PC-Hy or In-Hy during the first 4 d of culturing to generate antibody secreting clones, as defined by the criteria given in Materials and Methods. Although the yield of PNA^{high},sκ^{low}
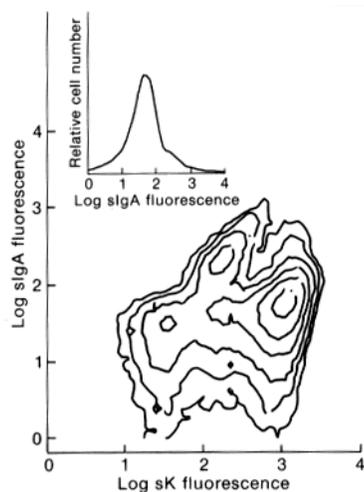
FIGURE 3. PP cells were stained with biotin–anti-IgA, FITC–anti-κ, and TR-avidin.
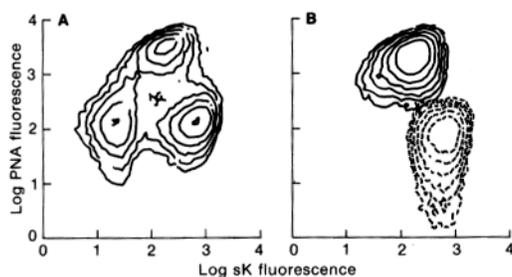

FIGURE 4. PP cells were stained with biotin-PNA, FITC–anti-κ, and TR-avidin. (A) Whole PP. (B) Analysis of separated subpopulations: PNA^{low},sκ^{high} and PNA^{high},sκ^{low}.

germinal center cells was usually threefold less than that of the PNA^{low},sκ^{high} cells, consecutive sorting and testing allowed 1.76×10^7 and 1.06×10^7 of the PNA^{high},sκ^{low} cells to be analyzed for PC- and In-specific responsive clonal precursors, respectively. Few, if any clones were generated by these B cells by our standard criteria, although a few fragment cultures tested positively for anti-PC or anti-In antibody, usually only on the first day of sampling (day 9 of culture). The antibody output of these positive cultures was usually far below that of conventionally defined clones and was almost always only IgM (31 of 35 positive fragments). Finally, such positive cultures were found at about the same incidence regardless of the presence or absence of antigen in the cultures. It should also be noted that most of these positive cultures would not have been regarded as clones in the usual splenic fragment assay such as those shown in Table I and that their generation is dependent on the donor cell inoculum since Hy-primed animals that receive no cells fail to yield positive fragment cultures.

Comparison of mRNAα Levels in PP B Cell Subpopulations. Since the IgA memory cells were found in the PNA^{low},sκ^{high} subpopulation, which contains mostly small B lymphocytes (Schweitzer, P. A., W. D. Ohriner, J. A. Cebra-Thomas, and J. J. Cebra, manuscript in preparation), attempts were made to detect mRNA for α chain in these cells and compare its level with that in germinal center B cells. Fig. 5 demonstrates that the majority of mRNAα in PP cells is found in the PNA^{high},sκ^{low} germinal center cells, but lower cells of mRNAα can be detected in the small B cells.

TABLE III

Peyer's Patch B Cells that Bind Low Levels of PNA (PNA^{low}) Account for Thymus-dependent Clonal Precursor Cells, Including IgA Memory Cells

Isotype expressed	Anti-PC*		Anti-In‡	
	PNA^{low}	Unsorted	PNA^{low}	Unsorted
			%	
Antigen in cultures				
Some IgA	68	53	77	88
IgM only	10	47	15	6
IgA only	32	20	23	64
IgG only	7	0	0	6
IgM + IgA	22	33	0	9
IgM + IgG	17	0	4	0
IgM + IgG + IgA	7	0	15	0
IgG + IgA	7	0	42	15
Number of clones analyzed	79	17	33	51
Frequency per 10^6 B cells§	57	24	32	46
No Antigen in Cultures‖				
Number of clones analyzed	0	2	2	3
Frequency per 10^6 B cells	<8	4	16	12

* Clones specific for PC represent data pooled from five separate experiments. Clones from PNA^{low} and unsorted PP cells were derived from 63.5 × 10^6 and 30 × 10^6 cells, respectively. PNA^{high}: 12 positive fragments of which 11 contained IgM only and 1 contained IgM + IgA were derived from 17.6 × 10^6 cells. The maximum frequency per 10^6 B cells is 17.

‡ Clones specific for inulin represent data pooled from three separate experiments. Clones from PNA^{low} and unsorted PP cells were derived from 42.5 × 10^6 and 46 × 10^6 cells, respectively. PNA^{high}: 23 positive fragments of which 20 contained IgM only, 2 contained IgA and IgG, and 1 contained IgA only were derived from 10.6 × 10^6 cells. The maximum frequency per 10^6 B cells is 55.

§ Frequency was determined as in Table I.

‖ PC and In-specific clones were derived from 5 × 10^6 PNA^{low} PP cells or 10^7 unsorted PP cells. PNA^{high} cells gave about the same incidence of antibody positive fragments regardless of the presence or absence of antigen.

Discussion

One unresolved aspect of B cell development is the relationship between surface isotype expression and isotype secretion by clonal progeny during antigen-specific expansion. If the deletion model, supported by analyses of myelomas and hybridomas is relevant (4–10), it would be expected that surface isotype expression and ultimate isotype secretion during clonal expansion are correlated. In fact a previous study demonstrated that expression of sIgG was associated with isotype restriction of clonal progeny to non-IgM isotypes (36). Since the Cα gene is the most 3' C$_H$ gene (37), sIgA$^+$ B lymphocytes would be expected to be restricted to IgA secretion. In this regard, PP were sorted on the basis of sIgA expression, and the isotype display of antigen-specific precursors was evaluated. The data presented in Table I demonstrate that clones derived from sIgA$^+$ PP B cells are restricted in isotype display to IgA secretion. Since the response to environmental determinants such as In is in itself restricted, i.e., the majority of antigen-specific precursors give rise to clones that secrete IgA exclusively (20),
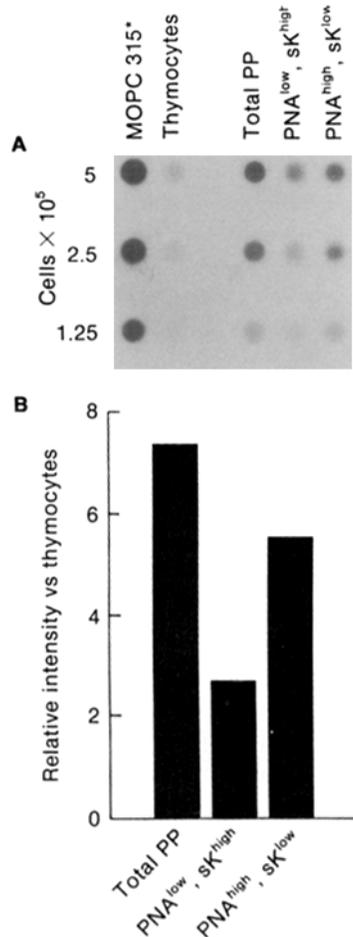
FIGURE 5. PP cells were stained with biotin-PNA, FITC–anti-κ, and TR-avidin and separated into the indicated subpopulations using a FACS IV. (A) Cytoplasmic dot blots probed for α-specific mRNA. (*) Serial dilutions of MOPC 315 start at 2.5 × 10⁵ cells. (B) Densitometry comparing 2.5 × 10⁵ cells from indicated populations with thymocytes.

it is important to note that the sIgA⁺ population is considerably enriched for In-specific precursors, and that all the isotype diversity can be accounted for by the sIgA⁻ population. These results provide the positive complement to our previous finding that the IgA memory cells from PP were sIgM⁻, sIgD⁻ (36).

Other investigators have provided evidence that the expression of non-IgM isotypes by B cells does not restrict isotype display during clonal expansion by demonstrating that the majority of sIgG⁺ B cells derived from antigen-primed spleens give rise to clones that secrete IgM (16). The conclusions of this study apparently contradict our own present and previous (36) findings that FACS enrichment of B cells from antigen-primed donors for cells bearing non-IgM isotypes also enriches for antigen-specific precursors and results in a marked increase in the proportion of these that generate clones that do not express IgM. However, the study by Lafrenz et al. (16) seems to contain some internal inconsistencies. In the initial experiment (16, 38), the majority of clones derived from spleens of mice primed to DNP, 77%, secreted IgG ± IgA without IgM, whereas in the second experiment clones of this sort were in the minority, 27%.

In addition, the results of the initial experiment (16, 38) are essentially consistent with our own, since $sIgG^+$ cells are enriched for precursors that give rise to clones secreting IgG ± IgA without IgM. The second part of the study used a population of B cells that are stripped of sIg with protease and cultured overnight to allow regeneration of sIg before sorting (16). We would suggest that the choice of B cell specificity, DNP, rather than the PC and In specificities we mainly probed, and the overnight culturing step, together with the limitations of enrichment of minority subpopulations by FACS, account for the inconsistencies in the Lafrenz et al. study (16) and the apparent discrepancies with our present and previous (36) analyses. We do not know whether secondary B cells expressing non-IgM isotypes regenerate levels of sIg after stripping comparable to their original surface density. However, Lafrenz et al. do acknowledge that their stripped, cultured cells did not show a clear inflection point between $sIgG^+$ and $sIgG^-$ cells of the kind we observed after staining for sIgA (Fig. 1). This technical problem results in considerable crosscontamination of the minority subpopulation as we have found for $sIgG1^+$ memory cells using FACS separation and based on data from enrichment of precursors (39). We would propose that the primed DNP-specific IgG^+ B cells evaluated by Lafrenz et al. (16) were initially in the minority of DNP-responsive clonal precursors, and after culturing were further diluted out by newly arising primary B cells of the high frequency DNP specificity as a result of pre-B cells undergoing sIg^- to sIg transitions in the cultured splenocytes (40,41). The FACS procedures used simply could not resolve the small minority of primed DNP-specific B cells (only 12% of clones from unfractionated, stripped, and cultured cells gave clones that did not express IgM; see reference 16) and hence all fractions tested gave statistically the same isotype profile: that of the majority DNP-specific B cells in the unfractionated, cultured population.

A second aspect of the relationship between surface isotype expression and isotype secretion is the question as to whether a B cell becomes committed to a secondary isotype before surface expression of that isotype. A cell line I.29 that does not normally express sIgA until stimulated with LPS has been described (42). Upon stimulation with LPS, this cell line preferentially expresses sIgA; however, before stimulation with LPS the cell line contains $mRNA\alpha$ (42). An apparently significant (39%) proportion of our $sIgA^-$ PP cells (Table I) gives rise to clones that are committed to IgA secretion, i.e., clones that secrete IgA exclusively, suggesting that isotype commitment in B cells may not necessarily be reflected by surface isotype expression. Although it has not been possible to detect $mRNA\alpha$ in the $sIgA^-$ population of PP cells by cytoplasmic dot blotting and the $sIgA^+$ population appears to account for most if not all of the $mRNA\alpha$ in PP, it is possible that a minority population synthesizing low levels of $mRNA\alpha$ cannot be detected by this technique. Thus, our observations suggest that B cells may become committed to a non-IgM isotype before its expression on the membrane.

The role of germinal centers in the generation of both memory cells and plasmablasts has been inferred by a variety of correlative studies (21–23, 43). In this regard, it would be expected that PP germinal centers are the source of both IgA memory cells and IgA plasmablasts. However, functional IgA-specific mem-

ory cells have not been detected in germinal centers using standard splenic fragment culturing in vitro (Table I). Other investigators have indicated that antigen-specific memory cells are preferentially found in the PNA^{high} germinal center population of draining lymph nodes 10 d after antigenic stimulation (44). Coico et al. (44) define memory cells as those cells that give rise to antigen-specific indirect PFCs in sublethally irradiated recipient animals 7 d after adoptive transfer of cells with antigen. However, this operational definition of memory cells does not distinguish between preplasma cells and memory cells. In addition, the experimental design does not demonstrate the ability of the putative memory cells to be stimulated by antigen in the host, an accepted property of memory cells (45). Given these considerations it seems critical to demonstrate in clonal cultures whether PP germinal center cells are the precursors for IgA memory cells found in the PP and whether they eventually display the phenotype $PNA^{low}, sIgA^+, s\kappa^{high}$.

Not only do the majority of PNA^{high} germinal center PP cells bear sIgA (29) but they also appear to be the PP subpopulation most active in synthesizing α chain (46) and with the highest content of $mRNA\alpha$ (Fig. 5). In addition, it has recently been shown using in situ hybridization (Schweitzer, P. A., W. D. Ohriner, J. A. Cebra-Thomas, and J. J. Cebra, manuscript in preparation) that PP germinal centers are enriched in a population of relatively large cells containing high levels of $mRNA\alpha$. Thus, it is likely that IgA PFCs specific for environmental antigens such as In and PC could be derived from PP germinal center cells using an assay system similar to that used by Coico et al. (44). However, in our study presented here, clonal expansion after antigenic challenge is being used as a criterion for memory cell precursors. It remains to be determined if it is possible to detect memory cells that are derived from germinal center cells if those cells are stimulated at a later time, i.e., after they have rested in a nongerminal center environment either in vitro or in vivo. Nonetheless, it could be argued that the inability to detect functional IgA memory cells in the PNA^{high} population using splenic fragment culturing is due to the relative inability of PNA^{high} PP cells to lodge in the spleen (47). However, when PP cells are separated into PNA^{high} and PNA^{low} subpopulations, the percentage of PNA^{high} cells that lodge in the spleen is at least half that of PNA^{low} cells (47). Furthermore, the fact that some fragment cultures derived from recipients that received PNA^{high} PP cells tested positively for antibody (Table III) suggests that it is not an inability to reach the spleen that is responsible for the inability to detect functional IgA memory cells in the PP germinal center population. Rather, any putative prememory population in germinal centers does not appear to be in a state from which it can be stimulated by antigen to give rise to IgA-secreting, antigen-specific clones. It remains to be determined whether the $sIgA^+$ cells of PP germinal centers have undergone C_H-gene deletions and/or rearrangement and whether they contain precursors for functional memory cells.

Summary

IgA memory B cells have been operationally defined as precursors that give rise to clones exclusively secreting IgA antibodies upon antigen stimulation in a T-cell dependent splenic fragment culture. B lymphocytes that are $sIgA^+$ account

for a small fraction of Peyer's patch lymphocytes, but these can be clearly divided into two subsets. One subset contains the majority of $sIgA^+$ B cells and most of these are in S, G_2, or M phase of the cell cycle. These cells are germinal center B cells, as defined by being $s\kappa^{low}$ and peanut agglutinin $(PNA)^{high}$, and contain most of the mRNAα. Though these germinal center cells may contain the majority of $sIgA^+$ B cells and may contain precursors for memory cells, preplasma cells, or both, they do not appear to be immediately responsive to stimulation by antigen. Rather, the $s\kappa^{high},PNA^{low}$ subset of $sIgA^+$ B cells, most of which are in G_0 or G_1 and have only low levels of mRNAα appear to contain most of the clonal precursors that are committed to IgA, i.e., the functional memory cells that give rise to clones exclusively secreting IgA upon stimulation with thymus-dependent antigen in the presence of T cells. There is also a population of Peyer's patch B cells that neither bears detectable sIgA nor has mRNAα detectable by cytoplasmic dot blotting but contains a small proportion of the functional IgA memory cells.

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