

RESTRICTION OF GENE EXPRESSION IN B LYMPHOCYTES AND THEIR PROGENY

II. COMMITMENT TO IMMUNOGLOBULIN HEAVY CHAIN ISOTYPE*

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Although the terminally differentiated plasma cell is committed to the synthesis of immunoglobulin (Ig) of a single heavy chain isotype (1, 2), it is not yet known whether the members of a clone of lymphoid cells are similarly restricted or whether they can switch from the synthesis of molecules of one isotype to another during their differentiation into plasma cells. A number of observations have been interpreted as showing that plasma cells within the same clone in some cases can secrete molecules of two different heavy chain classes (3, 4). In addition, the exposure of precursor lymphocytes to anti- μ chain antibodies can block the synthesis not only of IgM but also of IgG and/or IgA in chickens (5, 6) and mice (7–10), suggesting that plasma cells synthesizing all classes of Ig may be derived from precursor lymphocytes with IgM receptors on their membranes. The fact that the membrane Ig of the majority of B lymphocytes is IgM (11–13) while IgG and IgA molecules predominate in the serum and external secretions, respectively, also leads one to infer that IgM-bearing lymphocytes play a significant role in the generation of IgG- and IgA-producing immunocytes. However, in conflict with this notion are the results obtained in several other experimental systems indicating that the heavy chain isotype on a lymphocyte's membrane is the same as that which will be synthesized by its plasma cell descendants (14, 15).

We have previously shown that IgM is the only class of endogenous membrane Ig detectable by immunofluorescence techniques on lymphocytes from

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both Peyer's patches and lymph nodes in the rabbit (16). Despite this superficial similarity, cells from the two tissues were found to differ greatly in their potential to generate plasma cells making particular Ig heavy chain isotypes. Peyer's patch lymphocytes give rise to large numbers of IgA-producing plasma cells in the spleens and intestinal lamina propria of irradiated allogeneic recipients after cell transfer. In contrast, lymph node cells generate predominantly IgM and IgG plasma cells in the spleens of the recipients and are incapable of repopulating the gut with plasma cells (17, 18). The sum of these observations suggested that the precursors of the IgA plasma cells might be the IgM-bearing lymphocytes in the Peyer's patches.

To test whether IgM-bearing lymphocytes in the rabbit have the potential to differentiate into plasma cells synthesizing other isotypes of Ig, we took advantage of the ability of a fluorescence-activated cell sorter (FACS)¹ to separate those cells which have bound fluorescent molecules from the unstained cells (19, 20). In previous experiments with the FACS (21), lymphocytes separated according to their membrane *b* locus allotype gave rise to plasma cells synthesizing that same allotype, either in irradiated recipients or in vitro in the presence of pokeweed mitogen (PWM). In the studies reported here we have explored in a similar fashion the potentials of FACS-separated μ -bearing and μ -negative Peyer's patch and lymph node cells to generate IgA-, IgG-, and IgM-producing plasma cells.

Materials and Methods

Rabbits.—Rabbits homozygous for the b4 κ -chain allotype were obtained from commercial breeders. Homozygous *b*⁵/*b*⁵ rabbits were kindly provided by Dr. C. W. Todd, City of Hope, Duarte, Calif.

Preparation of Cells.—The preparation of single cell suspensions, the removal of membrane Ig with pronase, and the culture conditions for allowing the re-expression of membrane Ig have been described in a previous publication (21).

Immunofluorescence.—The preparation and specificity of the fluorescent antiallotype reagents have been described in detail (20). Briefly, rabbit anti-b5 antisera were raised by injecting b5 IgG into *b*⁴/*b*⁴ rabbits. IgG fractions of these sera were conjugated with fluorescein (F) to prepare F-labeled anti-b5 (F-anti-b5). Specific goat anti-b4 and goat anti-b5 reagents were also obtained and coupled with fluorochrome, yielding F-anti-b4, rhodamine (R) anti-b4, and R-anti-b5. The fluorochrome-coupled goat antirabbit heavy chain reagents also have been described (reference 16 and footnote 2).

Lymphocytes were membrane stained as detailed previously (21). Fixed smears or cyto-centrifuge preparations of the stained cells were examined; between 100 and 300 cells were counted/slide. Staining of cells for cytoplasmic Ig was done essentially as previously published (1, 21). To determine the proportion of total cytoplasmic Ig-stained cells (CSC) which were

¹Abbreviations used in this paper: CSC, cytoplasmic Ig-stained cell(s); F, fluorescein; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; μ +, μ -bearing; μ -, non- μ -bearing; PBS, phosphate-buffered saline; PWM, pokeweed mitogen; R, rhodamine.

²Craig, S. W., and J. J. Cebra. 1974. Rabbit Peyer's patch, appendix and popliteal lymph node B lymphocytes: a comparative analysis of their membrane immunoglobulin components and plasma cell precursor potential. Manuscript submitted for publication.

of each heavy chain class, a fluorescent antiallotype reagent specific for the *b* locus allotype of the cell donor was mixed with each contrastingly-labeled antiheavy chain reagent; each mixture was used to stain a separate slide. Only goat antiallotype reagents were used in conjunction with the goat anti- γ chain reagent because the latter would have reacted with the rabbit antiallotype reagents, which are IgG preparations. The relative proportions of CSC which were IgA, IgG, or IgM were determined on three slides, each stained with a mixture of antiallotype and the appropriate anticlass reagent. On each slide 300–400 *b* locus CSC were examined for their heavy chain isotype. The fluorescence microscope and the filters used for exciting and observing F and R fluorescence have been described (20, 22).

Cell Separation.—The FACS (19, 20) and the cell separation protocol (21) have been described fully in earlier publications.

Cell Transfer.—Cells from b^b/b^b rabbits were injected intravenously into lethally irradiated b^d/b^d rabbits, as detailed earlier (21). 1 wk after cell transfer the rabbits were sacrificed by exsanguination, and the spleens were removed and dissociated into single cell suspensions. Smears were prepared and stained with fluorescent antiallotype and anticlass-specific reagents to permit enumeration of IgA, IgG, and IgM CSC of donor origin. Between 8,000 and 50,000 cells were examined/slide to determine the proportion of total lymphoid cells which were donor CSC.

Microculture.—As described previously (21), cells were cultured with or without PWM in microcultures initially containing 2.5×10^5 viable cells. After 4 days the cells from quadruplicate cultures were pooled, centrifuged, and duplicate samples were taken for viable cell counts, using Trypan blue dye exclusion. Cytocentrifuge slides were prepared, fixed in ethanol, and stained with fluorescent antibody reagents to detect CSC and to identify the heavy chain isotype of their product Ig. Between 1,000 and 2,000 cells were examined/slide to determine the proportion of total lymphoid cells which were CSC.

RESULTS

Separation of Peyer's Patch and Lymph Node Cells Bearing Membrane μ -Chain.

—In the first two studies of whether lymphocytes bearing IgM give rise solely to cells staining for cytoplasmic IgM or also to cells containing IgA or IgG, cells from both lymph nodes and Peyer's patches were used. The sacculus rotundus, which is similar to the smaller Peyer's patches both morphologically (23) and in its precursor activity (17, 24), was pooled with the patches to provide sufficient numbers of cells for the experiments. The cells were first stripped of their membrane Ig with pronase to remove exogenously-bound Ig (20). In all experiments described in this paper, membrane staining immediately after pronase treatment verified that all detectable Ig had been removed. After overnight culture, which allowed lymphocytes to re-express their own membrane Ig, the cells were stained with F-labeled anti- μ chain (F-anti- μ) and passed through the FACS for separation into μ -bearing ($\mu+$) and non- μ -bearing ($\mu-$) populations.

In Table I are presented the proportions of the unseparated and separated cells which stained for membrane and cytoplasmic Ig before further culture or transfer, as observed in two experiments (nos. 1 and 2). The FACS gave excellent separations for μ -bearing cells; microscopic observation revealed that the $\mu+$ fractions consisted of 90% or more μ -bearing cells, while the $\mu-$ populations contained no detectable μ -bearing cells. Only a small proportion of total cells

TABLE I
*Separations for μ -Bearing Peyer's Patch and Lymph Node Cells: Membrane and Cytoplasmic Ig-Stained Cells in Unseparated and FACS-Separated Populations**

Cells	% μ -membrane stained	% CSC†	% (of total CSC)§		
			IgA	IgG	IgM
Peyer's patch					
Unseparated	35, 52	1.0, 1.2	42, 41	3, 0	53, 58
μ +	93, 93	1.4, 1.3	10, 12	0, 0	87, 86
μ -	0, 0	<0.1, 0.4	53, 100	33, 1	0, 0
Lymph node					
Unseparated	16, 21	0.2, 0.1	2, 5	54, 35	48, 57
μ +	90, 93	0.2, 0.1	0, 2	4, 4	88, 92
μ -	0, 0	<0.1, 0.1	0, 0	100, 89	0, 0

* Experiments 1 (b^5/b^5 cell donor) and 2 (b^4/b^4 cell donor).

† CSC, cytoplasmic Ig-stained cells; % CSC = (No. b4 or b5 CSC)/(no. total lymphoid cells) \times 100.

§ (No. CSC double stained for b4 or b5 and heavy chain isotype/No. total b4 or b5 CSC) \times 100. Determined on separate slides double stained, respectively, with R-anti-b4 or b5 plus F-anti- α , R-anti-b4 or b5 plus F-anti- γ , and F-anti-b4 or b5 plus R-anti- μ .

contained cytoplasmic Ig, 0.1–0.2% of lymph node cells and about 1% of Peyer's patch cells, as has been reported by others (25). Not all of the CSC detected by immunofluorescence are typical mature plasma cells; some are virtually indistinguishable from small lymphocytes by dark field microscopy but their cytoplasm stains brightly with fluorescent anti-Ig reagents. More than 40% of the Peyer's patch CSC were IgA; the remaining CSC were predominantly IgM. In contrast, 54% and 35% of the lymph node CSC were IgG in the two experiments. Most of the other CSC contained IgM, while only a few percent stained for IgA.

Whether or not the cells with cytoplasmic Ig after pronase stripping and overnight culture also had membrane IgM was determined by examining the segregation of CSC into either the μ +

Microculture of Peyer's Patch and Lymph Node Lymphocytes after Separation of Cells Bearing Membrane μ -Chain.—Samples of μ +

Ig were determined (Table II). It is readily apparent that PWM had a significant effect on unseparated Peyer's patch cells, increasing the numbers of viable cells per culture, the percentage of total cells which were CSC, and the proportion of total CSC which were IgA positive. In the presence of PWM, the $\mu+$ cells gave rise predominantly to IgM CSC, although some IgA CSC were found in these cultures. The majority of IgA CSC, however, arose from Peyer's patch cells which did not have membrane IgM, i.e., were $\mu-$.

Although PWM also enhanced the recovery of viable lymph node cells, it did not increase the percentage of total cells which were CSC, nor did it produce

TABLE II
*Separations for μ -Bearing Peyer's Patch and Lymph Node Cells: Recovery of Total and Cytoplasmic Ig-Stained Cells After Culture**

Cultures		No. viable cells/culture $\times 10^{-3}\ddagger$	% CSC§		% (of total CSC)§		
Cells	PWM				IgA	IgG	IgM
Peyer's patch							
Unseparated	-	122, 87	11, 6.5	11, 14	0, 0	82, 83	
Unseparated	+	153, 167	18, 11	60, 64	0, 0	41, 37	
$\mu+$	+	88, 22	15, 18	7.4, 5.0	0, 0	91, 97	
$\mu-$	+	65, 142	3.0, 3.8	97, 94	0, 1.0	0, 3.0	
Lymph node							
Unseparated	-	32, 69	23, 14	0.8, 2.3	0.8, 0.7	99, 91	
Unseparated	+	177, 114	12, 12	3.5, 3.0	2.0, 2.3	97, 97	
$\mu+$	+	109, 42	18, 53	1.8, 1.3	0.8, 1.0	100, 100	
$\mu-$	+	32, 32	0, 0	—	—	—	

* Experiments 1 and 2.

‡ Initial cultures contained 250×10^3 lymphoid cells.

§ See footnotes to Table I.

a dramatic shift in the proportions of CSC containing the different isotypes of Ig. With or without PWM present, greater than 90% of the CSC in cultures of unseparated cells were IgM; the precursors of these CSC were μ -bearing cells since the $\mu-$ population had not generated any CSC by day 4. Similarly, the few IgA and IgG CSC present after culture also may have been derived from cells with membrane IgM, assuming that the $\mu-$ cells in the $\mu+$ fraction would not behave differently from those in the $\mu-$ fraction.

To provide additional evidence that the CSC present after culture had descended from lymphocytes and not from the few CSC in the initial culture inocula, the magnitudes of the absolute increases in numbers of CSC per culture were determined. The numbers of CSC of each isotype per culture, calculated from the data in Table II, are given in Table III together with the numbers of CSC present before culture, obtained from the data in Table I. For the Peyer's patches the presence of PWM increased the numbers of IgA CSC arising in cultures of unseparated or $\mu-$ cells 5- to 16-fold during the 4 days of culture.

TABLE III
Separations for μ -Bearing Peyer's Patch and Lymph Node Cells: Levels of Cytoplasmic Ig-Stained Cells before and after Culture

Cultures		No. CSC/culture $\times 10^{-2}$ * (Before culture/after culture)					
		Exp. 1			Exp. 2		
Cells	PWM	IgA	IgG	IgM	IgA	IgG	IgM
Peyer's patch							
Unseparated	-	10/15	0.8/0	13/110	12/8.0	0/0	17/47
Unseparated	+	10/165	0.8/0	13/113	12/118	0/0	17/68
$\mu+$	+	3.5/9.8	0/0	30/120	3.8/2.0	0/0	28/39
$\mu-$	+	<1.3/19	<0.8/0	0/0	10/51	0.1/0	0/1.6
Lymph node							
Unseparated	-	0.1/0.6	2.7/0.6	2.4/73	0.1/2.2	0.9/0.7	1.4/88
Unseparated	+	0.1/7.4	2.7/4.2	2.4/206	0.1/4.1	0.9/3.2	1.4/133
$\mu+$	+	0/3.5	0.2/1.6	4.4/196	0.1/2.9	0.1/2.2	2.3/223
$\mu-$	+	<2.5/0	<2.5/0	<2.5/0	0/0	2.2/0	0/0

* Calculated from the data in Table I (before culture) and Table II (after culture).

Since the increase in numbers of CSC occurs abruptly, usually on the 4th day of culture (21, and unpublished observations), it is doubtful that increases of this magnitude would entirely have been due to the proliferation of previously existing CSC. Thus some de novo generation of CSC from lymphocyte precursors must have occurred. The magnitude of the increase in IgM CSC in cultures of unseparated or $\mu+$ Peyer's patch cells was more varied, ranging from 1.4- to 9-fold. (Recently it has been found that a different fetal calf serum supports much greater increases in numbers of IgM CSC.) The $\mu+$ cultures also contained some IgA CSC; their numbers, however, were too low to permit one to conclude that they had descended from μ -bearing precursor lymphocytes. Even if the IgA CSC counted on day 4 were the result of the differentiation of lymphocytes, the possible 7% contaminating $\mu-$ cells in the $\mu+$ population (cf. Table I) might have been sufficient to generate these IgA CSC.

For the lymph node, the numbers of IgM CSC increased markedly in cultures of unseparated or $\mu+$ cells both in the presence and absence of PWM; the increases ranged from 31- to 100-fold. Although the numbers of IgA and IgG CSC in cultures of $\mu+$ lymph node cells on day 4 were low compared to the numbers of IgM CSC, they were from 8 to at least 35 times greater than the number put into culture. Since the $\mu-$ lymph node cells did not give rise to any CSC, the few IgA and IgG CSC in the $\mu+$ cultures probably did not arise from contaminating $\mu-$ lymphocytes. Thus these IgA and IgG CSC were probably the descendants of $\mu+$ lymphocytes, again assuming that $\mu-$ cells behave the same in the presence of $\mu+$ cells as they do alone in culture.

Cell Transfer of Peyer's Patch and Lymph Node Lymphocytes after Separation of Cells Bearing Membrane μ -Chain.—An additional method for assessing the proliferative and differentiative potential of rabbit lymphocytes is the transfer of cells from a donor of one allotype into lethally irradiated allogeneic recipients

of a different allotype (26). To determine whether the CSC generated in both the microcultures and the cell transfer recipients have similar isotype distributions and precursor cell origins, in experiment 1 some unseparated and separated cells were also transferred into lethally irradiated b^4/b^4 recipients. As shown in Table IV, unseparated and $\mu-$ Peyer's patch lymphocytes yielded large numbers of CSC which were predominantly IgA. The $\mu+$ cells appeared to be less efficient in repopulating the recipients. One of the two rabbits which had received $\mu+$ Peyer's patch cells (no. 4) had no b5 CSC in his spleen, and

TABLE IV
*Separations for μ -Bearing Peyer's Patch and Lymph Node Cells: Levels of Cytoplasmic Ig-Stained Cells in Spleens of Cell Transfer Recipients**

Recipient no.	Tissue	Inoculum		% (of total CSC)†			Degree of repopulation‡
		Cells	No. $\times 10^{-6}$	IgA	IgG	IgM	
1	Peyer's patch	Unseparated	4.0	100	0	0	++
2		Unseparated	4.0	100	0	0	++
3		$\mu+$	4.0	100	1	0	\pm
4		$\mu+$	4.0	0	0	0	—
5		$\mu-$	4.0	100	1	0	++
6	Lymph node	Unseparated	1.0	2	7	86	++
7		Unseparated	1.0	5	29	68	++
8		$\mu+$	1.0	0	10	89	++
9		$\mu+$	1.0	0	3	97	++

* Experiment 1.

† See footnote to Table I.

‡ Qualitative estimation of frequency of b5 CSC on smears prepared from recipients' spleen cells.

the spleen of recipient 3 had considerably fewer donor CSC than did recipients 1, 2, or 5; these CSC were IgA. Since as few as 2.5×10^5 $\mu-$ cells are sufficient to repopulate recipients' spleens (see below), the low number of IgA CSC in recipient 3's spleen could have originated from the contaminating 7% $\mu-$ cells. Thus, in contrast to the microculture results, the $\mu+$ Peyer's patch cells did not seem to produce IgM CSC in this in vivo assay; even in vitro they were probably not as efficient as were the $\mu+$ lymph node cells at generating plasma cells.

In contrast, $\mu+$ cells from lymph nodes were very active after transfer, resulting in large numbers of donor-derived IgM CSC in the recipients. As in the microcultures, the $\mu+$ population also gave rise to some IgG CSC, but because there were no surviving recipients of $\mu-$ lymph node cells we could not

ascertain whether they might have been derived from contaminating μ^- cells conceivably able to function as IgG precursor cells in the transfer system.

It is apparent that neither the microculture nor the cell transfer techniques stimulated the appearance of large numbers of IgG CSC from either Peyer's patch or lymph node cells, so it was considered unlikely that either system would give us extensive information concerning whether μ -bearing lymphocytes could give rise to daughter IgG CSC. As a result, we decided to concentrate on probing the cellular origin of the IgA CSC arising from Peyer's patch lymphocytes.

*Separation of Peyer's Patch Cells Bearing Membrane μ -Chain and *b* Allotype Determinants.*—In earlier experiments in which lymphocytes from b^5/b^9 heterozygous rabbits were separated by the FACS into b^5 -bearing and b^9 -bearing populations for the study of commitment to allotype, we had found that the cells without detectable membrane *b* locus determinants generated very few CSC in microculture. An average of only 0.27% of the cells in cultures of b -negative Peyer's patch lymphocytes stained for cytoplasmic Ig after 4 days in culture with mitogen (21). This low yield of CSC from light chain-negative cells contrasts sharply with the results obtained when μ^- cells were cultured; in experiments 1 and 2, 3.0% and 3.8%, respectively, of the total cells were CSC (Table II). These results suggested that the precursors of IgA CSC are a class of Peyer's patch lymphocytes which can be stained by the fluorescent anti-*b* locus reagents but which do not have detectable μ -chain determinants.

Taking advantage of the double-pass cell separation procedure developed to allow the separation of cells bearing two different allotypes from populations of lymphocytes from heterozygous rabbits (20), we attempted to isolate a class of cells which had *b* locus light chain determinants but not μ -chain on their membranes. Peyer's patch lymphocytes from b^5/b^5 rabbits, after having been stripped with pronase and allowed to regenerate their membrane Ig, were stained with F-anti- μ and separated by the FACS into $\mu+$ and μ^- populations as before. The μ^- cells were then stained with R-anti- b^5 and repassed through the FACS, generating μ^-,b^5+ and μ^-,b^5- fractions. In addition, a sample of the unseparated cells was double stained with F-anti- μ and R-anti- b^5 and examined under the microscope to determine the membrane Ig markers present on the unseparated cells. Table V shows the proportion of cells which stained for membrane and cytoplasmic Ig before culture or transfer in experiments 3 and 4. In the unseparated cell preparations 8% of the total lymphoid cells in both experiments were stained by the R-anti- b^5 but not by the F-anti- μ reagent. The majority of membrane stained lymphocytes, 34% and 38% of total cells, however, had both μ -chain and b^5 light chain determinants.

From Table V it can be seen that the distribution of CSC of the three heavy chain isotypes into the $\mu+$ and μ^- populations was essentially the same as it had been in experiments 1 and 2 (Table I). Nearly all the IgM CSC had membrane IgM, and again a significant proportion, 6% and 10% of the μ -bear-

ing CSC, contained IgA. When the μ^- cells were further fractionated into those with and without membrane *b* locus determinants, most of the IgA CSC and the few IgG CSC in experiment 4 were found in the $\mu^-, b5+$ fraction and hence had membrane Ig.

*Microculture of Peyer's Patch Lymphocytes after Separation of Cells Bearing Membrane μ -Chain and *b* Allotype Determinants.*—To determine which class of μ^- cells, either those with or those without membrane light chain determinants, contained the precursors of the IgA CSC, samples of the FACS-separated populations were put into culture with or without PWM. Table VI gives the

TABLE V
*Separations for μ^- and *b5*-Bearing Peyer's Patch Cells: Membrane and Cytoplasmic Ig-Stained Cells in Unseparated and FACS-Separated Populations**

Cells	% Membrane stained†			% CSC‡	% (of total CSC)§		
	μ	<i>b5</i>	$\mu b5$		IgA	IgG	IgM
Unseparated	0, 0	8, 8	34, 38	1.0, 0.6	43, 51	0, 0	55, 52
μ^+	95, 95	ND	ND	3.4, 0.7	10, 6	0, 0	92, 97
μ^-	0, 0	25, 30	0, 0	0.7, 0.2	95, 98	0, 3	6, 0
$\mu^-, b5+$	0, 0	91, 85	0, 0	0.2, 0.5	96, 98	0, 3	0, 0
$\mu^-, b5^-$	0, 0	0, 1	0, 0	0, 0.2	2/3, 4/4	0/0, 0/4	0/0, 0/1

* Experiments 3 and 4 (both cell donors were *b^h/b^h*).

† Samples of unseparated and separated cells were stained with both F-anti- μ and R-anti-*b5*.

‡ See footnotes to Table I.

TABLE VI
*Separations for μ^- and *b5*-Bearing Peyer's Patch Cells: Levels of Cytoplasmic Ig-Stained Cells before and after Culture*

Cells*	PWM	No. CSC/culture $\times 10^{-2}$ † (before culture/after culture)					
		Exp. 3			Exp. 4		
		IgA	IgG	IgM	IgA	IgG	IgM
Unseparated	—	11/12	0/0	14/39	ND	ND	ND
Unseparated	+	11/100	0/0	14/45	ND	ND	ND
μ^+	—	8.5/1.2	0/0	78/45	1.1/0.8	0/0	18/20
μ^+	+	8.5/2.3	0/0	78/75	1.1/2.0	0/0	18/38
μ^-	—	17/4.9	0/0	1.1/0.4	0.3/155	0/0	5.4/3.2
μ^-	+	17/96	0/0	1.1/0.9	0.3/438	0/0	5.4/13
$\mu^-, b5+$	+	4.8/98	0/1	0/1	12/82	0.4/0	0/0
$\mu^-, b5^-$	+	0/0	0/0	0/0	0.4/0.2	0/0	0/0
Mixtures:							
30% μ^+ /70% $\mu^-, b5^-$	+	2.6/0.7	0/0	23/9.5	.36/17	0/0	5.4/77
30% $\mu^-, b5+$ /70% $\mu^-, b5^-$	+	ND	ND	ND	4.0/336	0/0.12	0/3.4

* All cell populations were stained with both F-anti- μ and R-anti-*b5* before being put into culture.

† Calculated from the viable cell recovery, % CSC, and % of total CSC which were IgA, IgG, and IgM.

numbers of IgA, IgG, and IgM CSC per culture initially present on day 0 and also on day 4. As had been found in the previous two experiments, the presence of PWM markedly stimulated the appearance of IgA CSC in cultures of unseparated or μ^- cells, here resulting in absolute increases in numbers of IgA CSC up to about 1,500 times the number present on day 0 (exp. 4). When the μ^- population was fractionated into $\mu^-,b5^+$ and $\mu^-,b5^-$ cells, IgA CSC were found only in cultures of $\mu^-,b5^+$ cells. Thus the precursors of IgA CSC do have membrane Ig, but it does not seem to be IgM. This unique class of Peyer's patch cells, found to comprise only 8% of total cells in these two experiments and 2% and 12%, respectively, in two other staining experiments, contains the cells which are specifically stimulated by PWM to give rise to IgA CSC.

As in experiments 1 and 2, the presence of PWM increased the numbers of IgM CSC only slightly in unseparated and μ^+ cultures. The presence of some IgA CSC in cultures of μ^+ cells, and of IgM CSC in cultures of μ^- cells again is difficult to evaluate, but there were too few to allow one to conclude that they were derived from lymphocytes with membrane Ig of different isotypes.

Occasionally in these experiments the CSC yield in cultures of FACS-separated populations was not as high as would have been expected from the number of CSC present in cultures of unseparated cells. For example, in experiment 4 (Table VI) cultures of μ^- lymphocytes, of which 30% membrane stained initially for b5 determinants, gave rise to 43,800 IgA CSC in the presence of PWM, but the $\mu^-,b5^+$ FACS fraction produced only 8,200 IgA CSC/culture even though it had been enriched to 85% for b5+ cells. Analogous observations had been made in experiments involving cells from heterozygous rabbits separated according to membrane allotype (21). In those studies it had been found that the addition of b- cells to cultures of b5+ or b9+ cells raised the numbers of CSC present after 4 days to the levels in the cultures of unseparated cells. The CSC were all of the same allotype as the membrane Ig of the b+ cells, demonstrating that the b- cells had not become functional precursors of CSC under those conditions.

Because these findings suggested that there might be synergy between different populations of Peyer's patch lymphocytes leading to an enhanced appearance of CSC, μ^+ and $\mu^-,b5^+$ cells were mixed with $\mu^-,b5^-$ cells in a ratio of 40% Ig+/70% Ig-, which was chosen because it approximated the relative proportions of these classes in both the unseparated and μ^- populations (cf. Table V). Although in experiment 3 (Table VI), the yield of IgM CSC dropped in the 30% μ^+ /70% $\mu^-,b5^-$ cultures relative to cultures of μ^+ cells alone, in experiment 4 the addition of $\mu^-,b5^-$ cells to μ^+ and to $\mu^-,b5^+$ cells increased the numbers of IgM and IgA CSC, respectively. Whether these effects were due to cell interactions between Ig+ and Ig- cells, to nonspecific soluble factors released by PWM-stimulated cells acting on CSC precursors, or merely to improved culture conditions caused by the presence of

one particular class of cells, is unknown. However, the net result was an improvement in the CSC recovery so that it was closer to the levels anticipated from the initial content of $\mu+$ or $\mu-,b5+$ cells in the cultures.

Cell transfer of Peyer's Patch Lymphocytes after Separation of Cells Bearing Membrane μ -Chain and b Allotype Determinants.—To compare the potential of the $\mu-,b5+$ and $\mu-,b5-$ Peyer's patch lymphocytes to differentiate into IgA CSC after allogeneic cell transfer, some of the cells separated by the FACS in experiment 3 were transferred into b^4/b^4 recipients. 1 wk later smears of the recipients' spleen cells were double stained with R-anti-b5 and F-anti- μ to determine the numbers of donor allotype CSC present and the proportions of these synthesizing IgA. As shown in Table VII, neither the $\mu+$ nor the $\mu-,b5-$ lymphocytes generated significant numbers of b5 CSC. All of the precursors for the IgA CSC were in the $\mu-,b5+$ population, paralleling the results obtained in culture. But again none of the recipients had any IgM CSC, in contrast to the situation in vitro where IgM CSC were found in cultures of unseparated and $\mu+$ lymphocytes.

In summary, aside from this difference in the ability of $\mu+$ Peyer's patch cells to generate IgM CSC in vitro and in vivo, the data obtained with both assay systems are in agreement. For both Peyer's patch and lymph node cells, μ -bearing lymphocytes give rise to all the IgM CSC but only to very low numbers of IgA and IgG CSC. In addition, the unique ability of Peyer's patch lymphocytes to generate large numbers of IgA CSC lies in a small population of cells which

TABLE VII
*Separations for μ - and b5-Bearing Peyer's Patch Cells: Levels of Cytoplasmic Ig-Stained Cells in Spleens of Cell Transfer Recipients**

Recipient no.	Inoculum		No. b5 CSC/10 ⁶ total cells	% IgA (of total b5 CSC)§
	Cell†	No. × 10 ⁻⁶		
10	$\mu+$	5.0	48	— (0/1)
11	$\mu+$	5.0	60	— (2/3)
12	$\mu+$	5.0	0 (<28)	— (0/0)
13	$\mu-$	2.5	430	100
14	$\mu-$	2.5	960	100
15	$\mu-$	5.0	1030	99
16	$\mu-, b5+$	2.5	6600	100
17	$\mu-, b5+$	2.5	930	100
18	$\mu-, b5-$	5.0	0 (<26)	— (0/0)
19	$\mu-, b5-$	5.0	0 (<26)	— (0/0)

* Experiment 3.

† All cell populations were stained with both F-anti- μ and R-anti-b5 before being transferred.

§ See footnote to Table I.

have *b* locus light chain determinants on their membranes but no detectable μ -chains; we have called these cells the immediate precursors of IgA plasma cells.

DISCUSSION

In our studies with FACS-purified populations it is probable that the majority of CSC present in recipients' spleens 7 days after transfer and in microcultures after 4 days were the result of the proliferation and/or differentiation of precursor lymphocytes. Very few donor-derived CSC are evident in recipients' spleens before day 6, after which there is an explosive appearance of CSC (S. Craig and P. Jones, unpublished observations). The organization of these CSC in large densely packed clusters or foci in the splenic red pulp (26) suggests that the precursors have proliferated before completing their maturation into plasma cells. Similar events probably also take place in the microcultures, as shown by the absolute increases in numbers of CSC per culture, which usually take place in the final 1 or 2 days of the culture period.

Although the μ -chain had been shown to be the only heavy chain in endogenous membrane Ig on rabbit lymph node and Peyer's patch lymphocytes (reference 16 and footnote 2), double fluorescent antibody staining revealed a minor population of cells which have light chain (*b* locus) determinants but no detectable μ -chain. These cells, isolated from the others with the FACS, were shown to contain all the functional precursors for the IgA CSC generated either *in vitro* in the presence of PWM or *in vivo* in irradiated recipients.

Even though our specifically purified goat antirabbit α -chain antibody reagent did not react detectably with membrane determinants (reference 16 and footnote 2), it is probable that the Ig present on these μ -, *b*+ lymphocytes is IgA. Not only does this population of cells include all the precursors of IgA CSC and no precursors of IgM or IgG CSC, but it also contains most of the IgA CSC in the Peyer's patch, which thus bear non-IgM Ig on their membranes. The apparent lack of reactivity of our goat anti- α chain reagent in fluorescent antibody staining may be due to its specificity being for determinants on the Fc portion of the α -chain, a part of the molecule which may be buried in the membrane. In recent immunofluorescence studies we have in fact been able to detect α -chain markers on some lymphocyte membranes, using antibodies directed against the *f* and *g* locus α -chain allotypic markers.³ Some of the determinants controlled by these loci are in the Fd portion of the α -chain (27) and thus may be available for the binding of antibody. For membrane-associated γ -chains, determinants in the Fd region have also been found to be more accessible to antibody reagents than those in the Fc (12, 28).

Both because different anticlass-specific reagents may not have equal opportunities to react with membrane Ig and because serum Ig can bind non-

³ Jones, P. P., and J. J. Cebra. Manuscript in preparation.

specifically to the surfaces of lymphocytes (20, 29–31), membrane-staining analyses must be supplemented with functional studies in order to ascertain whether or not the isotype of a lymphocyte's membrane Ig is the same as that secreted by the cell's plasma cell descendents. In the past, such functional tests have involved the use of antiheavy chain antisera to inhibit the synthesis of Ig in vivo or in vitro. In mice, exposure of cells to anti- μ antibodies blocked the synthesis of IgM, IgG, and IgA, whereas anti- α and anti- γ antisera selectively eliminated only IgA and IgG, respectively (7–10). In similar experiments in chickens, embryonic treatment with anti- μ antiserum combined with bursectomy at hatching prevented the synthesis of all three classes of Ig (5, 6). These results were interpreted as suggesting that plasma cells synthesizing IgA, IgG, and IgM all are derived from precursors bearing IgM receptors on their membranes, and that during their differentiation to IgA- or IgG-producing cells the α - and γ -chains also are expressed.

However, because of the possibility that the effect of the anti- μ antibodies is not a direct one on the B-cell precursors but perhaps is on some other cell or process required for antibody synthesis, the conclusion that there is a common μ -bearing precursor cell for plasma cells of all classes is still open to some question. For example, if T cells do have membrane IgM, as shown by some (32) though not all (13) investigators, then the anti- μ might exert its inhibitory effects on IgA and IgG synthesis by preventing effective cooperation between T and B cells.

The use of the FACS has permitted the direct identification of the heavy chain isotypes synthesized by the CSC descendents of specifically isolated lymphocytes bearing a particular class of membrane Ig. Although both the cell transfer and the microculture results were in agreement in demonstrating that $\mu+$ lymph node cells are the precursors of IgM CSC and that $\mu-, b+$ Peyer's patch cells are the precursors of IgA CSC, the two assay systems for precursor cell activity differentially stimulated the appearance of IgM CSC from Peyer's patch lymphocytes. In PWM-stimulated cultures of unseparated cells, approximately 40% of the CSC present on day 4 were IgM, and the $\mu+$ population also gave rise to significant numbers of IgM CSC. However, neither the unseparated nor the $\mu+$ Peyer's patch populations were able to generate IgM CSC after transfer into irradiated recipients. Thus the requirements for triggering the $\mu+$ Peyer's patch cells to differentiate or mature into IgM CSC are not met in the recipients, marking these cells as being different functionally from the lymph node $\mu+$ cells.

It is possible that the $\mu+$ Peyer's patch cells are too immature to respond immediately to the antigens present in the irradiated allogeneic hosts; this might be expected if Peyer's patches are the mammalian equivalent of the bursa of Fabricius (33). However, Peyer's patches have been shown to contain immediate precursors of IgM antibody-forming cells, in both in vitro (34) and in vivo (35) assays. An alternative explanation for the relative scarcity of IgM

CSC in the spleens of our recipients of Peyer's patch cells might be that the lymphocyte precursors of IgM CSC do not divide as extensively as do either the precursors of IgA CSC from the Peyer's patches or the IgM precursors from lymph nodes before their maturation into plasma cells. Such a difference in proliferative potential would result in fewer IgM than IgA CSC being present in the recipients' spleens 1 wk after the transfer of Peyer's patch cells.

On the whole our results suggest most strongly that the vast majority of lymphocytes in the Peyer's patches and lymph nodes are precommitted to the class of heavy chain that they and their daughter plasma cells will synthesize. However, a few observations support the possibility that a proportion of lymphoid cells are not so restricted to the synthesis of a single Ig isotype. Approximately 10% of the μ -bearing CSC in the Peyer's patches contain IgA in their cytoplasm; 4% of lymph node μ -bearing CSC contain IgG. Similar evidence for the presence of two different classes of Ig on the membrane and in the cytoplasm of plasma cells has been presented by Pernis et al. (36), who found that up to 15% of IgG plasma cells in the rabbit have membrane IgM. However, since those cells had not been previously stripped to allow the exclusive expression of endogenous Ig, the IgM may have been picked up by the cells. Rabbit serum IgM is capable of binding to the membranes of lymphoid cells, including plasma cells (P. Jones, unpublished observations). In the mouse, although about 50% of IgG CSC obtained directly from the spleen have membrane IgM, after overnight culture (without prior pronase stripping) at most a few per cent of IgG CSC bear membrane IgM.⁴ This decrease may be due to the loss of adherent IgM during the normal turnover of membrane components.

The appearance of some IgA or IgG CSC in cultures of μ + Peyer's patch or lymph node cells also suggests that not all lymphocytes are irreversibly committed to the synthesis of one isotype. In cultures of μ + lymph node cells the few per cent of total CSC which were IgA (1.3–1.8%) and IgG (0.8–1.0%) must have descended from μ + lymphocytes and not from contaminating μ - cells, since the latter did not contain any CSC precursors. It is more difficult to prove conclusively that the few IgA CSC present in μ + Peyer's patch cultures actually arose from μ -bearing cells because the μ + FACS fractions used were only 93–95% pure.

Based on the characteristics of various immunodeficiency diseases in humans (37) and on the inhibitory effects on IgA synthesis of bursectomy and anti- μ treatment in chickens (5, 6) and of anti- μ and anti- γ treatment in mice (8, 38), Cooper and his colleagues have proposed a "switch" hypothesis in which the sequence of Ig class expression within a clone is IgM \rightarrow IgG \rightarrow IgA (39). In our studies of the differentiative potential of Peyer's patch lymphocytes, we have found no evidence for the presence of significant numbers of IgG-synthesizing cells or their precursors. The immediate precursors of IgA CSC do not have

⁴ Eugster-Tacier, H., and L. A. Herzenberg. Manuscript in preparation.

membrane IgM, probably having IgA instead, and thus are distinct from the immediate precursors of IgM CSC. However, this conclusion is not incompatible with the possibility that these distinct cell lines may have diverged from common μ -bearing precursors earlier during ontogeny or in another lymphoid tissue, perhaps via a stage of IgG synthesis. It is also conceivable that the switch from the synthesis of IgM to non-IgM membrane Ig does occur in the Peyer's patches but that neither the cell transfer nor the microculture systems encourage the same differentiative processes that occur in situ.

The small population of μ -, b + lymphocytes, which confers on the Peyer's patch cells their unique ability to generate IgA CSC in irradiated recipients and in vitro in the presence of PWM, most likely is the source of the precursors for the vast arrays of IgA plasma cells in the lamina propria of the gut. The origins of these precursors, and the mechanisms by which they are stimulated to leave the Peyer's patches, lodge in the gut, and mature into IgA plasma are not yet understood. However, interaction of the precursors not only with antigen but also with nonspecific PWM-like stimulatory substances, such as endotoxin, may be essential for the generation of mature IgA antibody-forming cells.

SUMMARY

To determine whether or not B lymphocytes are committed to the synthesis of a single immunoglobulin heavy chain isotype during their differentiation into plasma cells, rabbit lymph node and Peyer's patch cells were separated into populations with and without membrane IgM, using a fluorescence-activated cell sorter (FACS). The potential of the μ -bearing (μ +) and non- μ -bearing (μ -) cells to give rise to plasma cells both in vivo after transfer into irradiated recipients and in vitro in the presence of pokeweed mitogen was assessed by immunofluorescence techniques, and the relative proportions of the cytoplasmic Ig-stained cells (CSC) synthesizing each class of heavy chains were determined.

Most of the CSC arising in vitro from μ -bearing lymph node and Peyer's patch cells contained IgM; all IgM CSC appeared to be derived from μ + cells. Peyer's patch lymphocytes, however, did not generate IgM CSC after cell transfer and thus may be functionally different from lymph node μ + cells. It was found also that nearly all of the many IgA CSC generated by Peyer's patch lymphocytes either in culture or after transfer were derived from μ - cells. Further fractionation of these μ - cells with the FACS after they had been membrane stained with anti-*b* locus allotype reagents revealed that the precursors of IgA CSC belong to a minor population of cells which do have *b* locus light chain determinants on their membranes, although they do not have detectable μ -chains. These cells are not found in lymph nodes.

Although the majority of Peyer's patch and lymph node cells were found to be precommitted to the synthesis of a single heavy chain isotype, a small proportion of cells may not be similarly restricted. Some of the CSC with membrane IgM were found to contain cytoplasmic IgA or IgG. In addition, μ + populations did give rise to low numbers of IgA and IgG CSC. The implications of

these results, obtained under experimental conditions, on the normal differentiation of B lymphocytes *in situ* are discussed.

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