RESTRICTION OF GENE EXPRESSION IN B LYMPHOCYTES AND THEIR PROGENY

I. Commitment to Immunoglobulin Allotype*

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The vast majority of lymphoid cells secreting completed immunoglobulin (Ig) show a striking restriction to the synthesis of Ig molecules of one class (1, 2), type (3, 4), allotype (5, 6), and specificity (7). While it is generally accepted that all cells within a clone synthesize molecules of the same specificity (7), there is less agreement as to whether all lymphoid cells within that clone produce the same isotype of Ig. From a few studies it may be inferred that bone marrow-derived (B) lymphocytes can synthesize a different isotype than their daughter plasma cells or that the different plasma cell members of a clone may produce different isotypes (8, 9). The knowledge of whether or not there can be changes in the allotype or isotype of the molecules produced by descendants of a single precursor cell will be important in understanding the regulation of gene expression in lymphoid cells.

With respect to allotype, there is conflicting evidence concerning the degree of restriction of lymphocytes and their descendant clonal elements to the synthesis of a single allelic product. Experiments utilizing the mixed antiglobulin technique (10) and blast transformation with antiallotype antisera (11) suggest that allelic exclusion might not be the rule for lymphocyte membrane Ig. Membrane staining with fluorochrome- or ^{125}I-labeled antibodies, however, has demonstrated that the vast majority of lymphocytes bear membrane receptors of only one allotype (12–14). Recently we have shown that rabbit lymphocytes are capable of binding exogenous serum Ig molecules (14) and have suggested that this property may explain the conflicting results from different laboratories.

A number of observations also suggest that there is commitment to the synthesis of one allotype during the differentiation of a lymphocyte with membrane Ig into antibody-secreting plasma cells. In allotype-suppressed rabbits (15, 16) and mice (17) exposure of fetal or neonatal heterozygous animals to antiallotype antibodies has suppressed the synthesis of molecules of that allotype, without reducing levels of the other allotype. This suppression of paternal allotype has been shown to be accompanied by a parallel depression in numbers of plasma cells containing the deficient kind of Ig in both suppressed rabbits (18) and mice (P. Jones, unpublished observa-

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tions). In addition, we have found previously that culturing peripheral blood lymphocytes from b^5/b^9 rabbits overnight in the presence of anti-b9 antibodies selectively stimulated the synthesis of b9 Ig after cell transfer into irradiated recipients, without affecting levels of b5 Ig (19). Finally, Bosma and Weiler (20) have shown that, after transfer of cells from mice heterozygous for allotypes of the γ2a-chain into irradiated recipients, the antipoly-D-alanine antibodies produced by individual splenic foci of cells were all of one allotype. These results imply that there is a divergence of precursor cells for the two allelic products at some stage during differentiation. However, this phenotypic restriction could occur in the progeny of lymphocytes already bearing membrane Ig of both allotypes. If the effects of treatment with antiallotype antibodies were on cells almost at the plasma cell stage, a lack of commitment earlier in the differentiation pathway would not have been revealed.

Using the fluorescence-activated cell sorter (FACS)[1] (21) we have been able to separate lymphocytes from rabbits heterozygous at the *b* (κ-chain) locus into populations purified for cells bearing membrane Ig of one or the other allotype (14). In a direct test of the commitment of lymphoid cells to allotype we have assessed the cells in such purified fractions for their ability to generate plasma cells synthesizing Ig of the same or alternative allotype.

Materials and Methods

Rabbits.—Rabbits homozygous for the b4 κ-chain allotype were obtained from commercial breeders. Heterozygous b^5/b^9 rabbits were either raised in our own facilities or were obtained from Dr. Rose Mage (Laboratory of Immunology. NIAID, NIH, Bethesda, Md.).

Preparation of Cells.—Peyer's Patch and peripheral blood lymphocytes were prepared as described previously (14). Peyer's Patch cells were obtained by teasing part the tissues at 4°C in minimal essential medium (MEM) (Grand Island Biological Co., Grand Island, N. Y., without sodium bicarbonate; 0.001 M in $MgCl_2$ and 0.001 M in NaH_2PO_4) containing 5% fetal calf serum (FCS) (GIBCO), 100 U/ml potassium penicillin, and 100 μg/ml streptomycin sulfate. The cells were passed through a stainless steel mesh and 6–8 ml of packed glass wool, washed twice, and counted in a hemacytometer.

To prepare peripheral blood lymphocytes, heparinized blood obtained from the marginal ear vein or by cardiac puncture was mixed with an equal volume of 3% gelatin in Ringer's solution. After allowing the erythrocytes to sediment at 37°C for 30 min, the supernate was centrifuged and the cells washed once at 25 or 37°C with medium (MEM containing 5% FCS, penicillin, and streptomycin) plus 5 U/ml heparin. Remaining erythrocytes were lysed by incubation for 3 min in 0.83% ammonium chloride in Gey's solution (minus sodium chloride). Peyer's Patch cells were 85–95% viable and peripheral blood cells were usually 95% viable, as judged by trypan blue exclusion. Giemsa staining indicated that cell preparations from both tissues routinely contained at least 95% lymphocytes.

Removal and Regeneration of Membrane Ig.—In some experiments cells were treated with pronase to remove their membrane Ig (14). Cells were suspended at 2–4 $\times$ 10^7 cells/ml in MEM without serum, to which was added an equal volume of 0.5% pronase (Pronase, Calbiochem., San Diego, Calif.) which had been preincubated at 37°C for 2 h, brought to pH 7.2,

[1] Abbreviations used in this paper: C, constant; CSC, cytoplasmic Ig-stained cell(s); F, fluorescien; F-anti-b5, fluorescein-labeled anti-b5; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FDA, fluorescein diacetate; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; PWM, pokeweed mitogen; R, rhodamine; R-anti-b9, rhodamine-labeled anti-b9; V, variable.

and filtered through a 0.22-μm Millipore filter before use. After incubation for 30 min at 37°C, the cells were centrifuged, incubated in 200 μg/ml DNase solution (Worthington Biochemical Corp., Freehold, N. J.) for 3 min at room temperature, and passed twice through a cushion of FCS before being counted in a hemacytometer. Cells were 95–100% viable after treatment, and the average viable cell recovery after stripping and subsequent processing was 84% of the number of cells present before pronase treatment.

To allow regeneration of membrane Ig, the cells were cultured in MEM with suspension salts (Microbiological Associates, Inc., Bethesda, Md.) containing 10% FCS (Rehatuin, Armour Pharmaceutical Co., Kankakee, Ill.), 100 U/ml penicillin, and 100 μg/ml streptomycin, at a concentration of 5×10^6 cells/ml, 35–45 ml/flask, in 75 ml screw-cap plastic culture flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). The cultures were equilibrated in 5% CO_2 in air, the caps were screwed tightly shut, and the flasks were incubated at 37°C on a rocking platform. After 16–18 h in culture the cells were harvested, and clumps were eliminated by incubation for 3 min at room temperature in 200 μg/ml DNase followed by rapid passage through 2 ml of packed glass wool. The cells were then washed once in MEM before being counted. The recovery of viable cells after culture and subsequent processing was 25–40% of the number of cells put into culture.

Immunofluorescence.—For membrane staining, $0.5–2.0 \times 10^7$ cells were centrifuged in siliconized 3-ml conical glass tubes and resuspended in 0.1 ml of either rhodamine-labeled anti-b9 (R-anti-b9) or fluorescein-labeled anti-b5 (F-anti-b5). The preparation and specificity of these reagents have been described previously (14). After 30 min incubation at 4°C the cells were washed once with medium and then layered over a 1.5-ml cushion of FCS and centrifuged. Smears or cytocentrifuge preparations (22) of the cells were fixed in 95% ethanol, air-dried, and mounted in a 9:1 mixture of glycerol and phosphate-buffered saline (PBS) under a coverslip for observation. Between 100 and 300 cells were examined/slide.

Staining of cells for cytoplasmic Ig was done as previously published (1). Smears or cytocentrifuge preparations were fixed in 95% ethanol, a drop of fluorescent antibody reagent was spread over the cells, and the slides were incubated for 45 min at room temperature in moist chambers. The slides were then washed for 10 min in PBS, fixed again in 95% ethanol, and mounted as described above. Between 1,000–2,000 cells were examined for cytoplasmic Ig-stained cells (CSC); relative proportions of b5 and b9 CSC were determined on counts of 300–400 total CSC.

The fluorescence microscope and filters used for exciting and observing fluorescein and rhodamine fluorescence have been described (14, 23).

FDA Staining.—Living cells were rendered fluorescent by the fluorogenic compound fluorescein diacetate (FDA), basically following the method of Rotman and Papermaster (24). To $1–2 \times 10^7$ cells in 1 ml of medium was added 2 μl of 5 mg/ml FDA (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.), dissolved in acetone. After 20 min incubation at room temperature the cells were centrifuged, passed twice through 1.5 ml FCS, and resuspended in medium.

Cell Separation.—Membrane stained cells were suspended at $5–10 \times 10^6$ cells/ml in Dulbecco's PBS containing 5% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin, and passed through the FACS at a rate of approximately 1.8 ml/h. Descriptions of the FACS have been published previously (21, 14). Briefly, the cells are illuminated by an argon ion laser for fluorescence excitation and by a helium neon laser whose light, after scattering from cells at small forward angles, gives an indication of particle size. Fluorescence and scattered light are detected by photomultiplier tubes, generating voltage pulses which, if falling within preset limits, lead to the deflection of the cells. The setting of thresholds for both fluorescence and scatter deflection will be discussed more fully below.

Cell Transfer.—Cells from b^5/b^9 rabbits were injected intravenously into b^4/b^4 rabbits which had received 800 R irradiation 25–30 h previously from a Philips 250 kV x-ray apparatus (Philips Electronic Instruments, Mount Vernon, N. Y.). Recipients were given 200,000

U potassium penicillin and 100 mg streptomycin sulfate daily, commencing the day before irradiation. 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 reagents to permit enumeration of b5 and b9 CSC.

Microcultures.—Cells were suspended at 1×10^6 cells/ml in RPMI 1640 medium (GIBCO) supplemented with 20% FCS (Rehatuin, Armour Pharmaceutical Co.,), 2.0 mM glutamine, and 50 μg/ml gentamicin sulfate (Microbiological Associates, Inc.). In some experiments phytohemagglutinin (PHA) (PHA-P, Difco Laboratories, Detroit, Mich., code no. 3110, reconstituted with 5 ml sterile distilled water), or pokeweed mitogen (PWM) (GIBCO, catalog no. 536, reconstituted with 5 ml sterile distilled water) was added to the suspension. 0.25 ml (2.5×10^5 cells) was placed in each well of Microtest II tissue culture plates (Falcon Plastics), and the plates were incubated in 5% CO_2 in air at 37°C for the desired period of time, usually 4 days. After culture, the cells from quadruplicate cultures were pooled, washed once, and duplicate samples were taken for the determination of the viable cell recovery. Cytocentrifuge slides were prepared and stained with fluorescent antibody reagents to detect CSC and to identify the allotype of their products.

RESULTS

FACS Pulse-Height Distributions.—During passage through the FACS, each cell or particle generates one signal proportional to its fluorescence intensity and another signal proportional to its low angle light-scattering properties, which give an indication of particle size. Pulse-height distributions, representing the accumulation of impulses for a large number of cells, indicate the relative proportions of cells with different scatter or fluorescence intensities and are useful in setting the minimal and maximal voltage values which will be used as thresholds for deflection.

Fig. 1 *a* shows a typical set of fluorescence pulse-height distributions for three separate samples of Peyer's Patch lymphocytes. In the cell preparation stained with R-anti-b9, there are some cells which are detected as being brighter than the cells in the unstained preparations. After separation of those cells defined as b9 positive (b9+), the b9 negative (b9−) cells were stained with F-anti-b5 and passed through the FACS. The pulse-height distribution of this sample clearly shows a discrete population of cells giving signals distinctly stronger than the unstained population. A larger proportion of cells appear to be brightly stained in the F-anti-b5-stained sample than in the R-anti-b9-stained sample, perhaps due to the higher proportion of stained cells; 38% of the cells were detected by eye as b5+, using the fluorescence microscope, compared with only 17% scored by eye as b9+ in the R-anti-b9-stained preparation. Alternatively, the intensity of fluorescence of the cells binding F-anti-b5 simply may register as being brighter than the fluorescence of the cells binding R-anti-b9. In fact, the sensitivity of the FACS for the fluorescence of fluorescein (F) conjugates is somewhat greater than that for rhodamine (R) conjugates. For both samples the thresholds were set so that cells which were brighter than the background fluorescence in the unstained sample would be deflected into one tube; the dim residual cells were deflected into a second tube. In later experiments improved electronics permitted two thresholds to be set, creating a narrow "window" of

intermediate voltage values. Cells generating impulses included within this window were collected into a third tube. Thus, "borderline"-positive cells, which were barely above background fluorescence and which might have introduced ambiguities into the experiments, could be eliminated.

Since fluorescent noncellular pieces of debris often are present in cell suspensions, the ability to detect and separate only those particles of cellular dimensions is important in obtaining accurate fluorescence pulse-height distributions

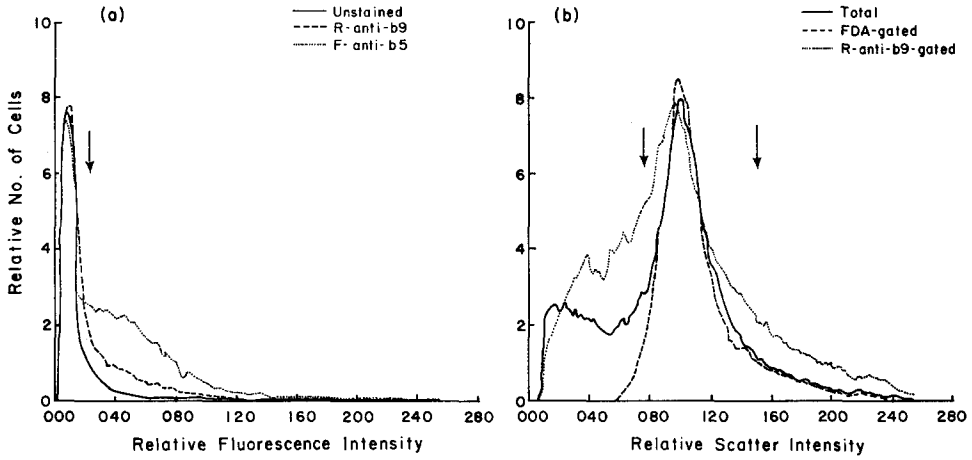

FIG. 1. Pulse-height distributions of Peyer's Patch lymphocytes. (*a*) Fluorescence. Lymphocytes from a b^5/b^9 rabbit were stained with R-anti-b9 and separated by the FACS into b9+ and b9− fractions. The latter was then stained with F-anti-b5 and passed through the cell sorter. A sample of unstained cells was also passed through the FACS to generate a profile due to background fluorescence. Pulse-height distributions for all three samples are given. The units on the abscissa correspond to channel numbers, increasing channel number indicating greater fluorescence intensity. The arrow indicates where the threshold for deflection was set. (*b*) Scatter. The R-anti-b9 stained sample was passed through the FACS. The scatter distribution of all cells and particles (total scatter) was generated, as was the scatter distribution of only the cells and particles which had bound R-anti-b9 and were above the fluorescence threshold (R-anti-b9-gated scatter). In addition, another cell sample was stained with FDA and the scatter distribution of the cells which were fluorescent was obtained (FDA-gated scatter). The units on the abscissa correspond to channel numbers, increasing channel number indicating greater scatter intensity and hence larger particles. The arrows indicate where thresholds were set between which deflection was permitted.

and good purification of the different cell populations. Fig. 1 *b* presents pulse-height distributions of the scatter intensities of samples from the experiment just described. The scatter profile generated by all the particles in the R-anti-b9-stained sample (total scatter) shows a typical bimodal distribution. The scatter profile of just those particles within the sample which were detected as being fluorescent (R-anti-b9-gated scatter) indicates that the fraction of the smaller particles which is stained is greater than that of the larger ones.

In exploring the meaning of this often observed segregation of lymphoid cells into two populations according to their scatter, it was found that the "peak" of smaller particles almost exclusively represents dead cells.[2] This analysis was established by the use of fluorochromasia (24); only living cells are rendered fluorescent by F diacetate (FDA). As shown in Fig. 1 *b*, when the scatter profile of those particles which fluoresce after FDA staining is generated (FDA-gated scatter), only cells represented by the second peak of scatter intensities are stained, indicating that few, if any, living cells contribute to the first peak. Dead cells, however, nonspecifically bind fluorescent antibodies, explaining the apparent higher proportion of R-anti-b9-stained cells in the first peak.

To prevent dead cells, as well as large and small pieces of debris, from being considered for deflection by the FACS, scatter thresholds can be set just above and below the principle mode so that only living cells registering within that peak are deflected. Thus, by setting appropriate thresholds for both fluorescence and scatter on the basis of the pulse-height distributions, clean and viable cell populations totally depleted or enriched to 95–100% for membrane stained cells can be obtained routinely. These cells can then be tested for their ability to give rise to plasma cells producing b5 or b9 Ig.

Effect of Prior Membrane Staining of Donor Lymphocytes on Their Repopulation of Recipients' Spleens.—An allogeneic rabbit-cell transfer system previously has been utilized to assess the potential of lymphocytes to differentiate into plasma cells (19, 25). Before this in vivo assay could be used to determine whether lymphocytes and their progeny are committed to the synthesis of molecules of a single allotype, it was necessary to demonstrate that membrane staining did not affect the functional capabilities of the transferred cells. Peyer's Patch lymphocytes from a b^5/b^9 rabbit were membrane stained with R-anti-b9 and F-anti-b5, then were maintained on ice for 6 h to mimic the treatment of cells during a separation experiment, and finally were transferred into lethally irradiated b^4/b^4 recipients. Other recipients received unstained cells from the same donor rabbit. Table I indicates that prior membrane staining does not prevent lymphocytes from differentiating to CSC. The apparent slight stimulatory effect of staining on the numbers of CSC is within the normal variation observed in this cell transfer system which utilizes outbred rabbits as recipients. The relative proportions of b5 and b9 CSC in the recipients also were not altered by treatment with the fluorescent antibody reagents, indicating that the two reagents do not differentially stimulate or inhibit the cells. The preponderance of b5 CSC is characteristic of the relative proportions of b5 and b9 serum molecules and plasma cells in b^5/b^9 rabbits (19, 26).

Transfer of Lymphocytes Separated According to Membrane Allotype.—We

[2] Julius, M. H., R. G. Sweet, C. G. Fathman, and L. A. Herzenberg. 1974. Fluorescence-activated cell sorter and its applications. First Los Alamos Life Sciences Symposium. AEC. In press.

have shown that when Peyer's Patch lymphocytes from b^5/b^9 rabbits are membrane stained for allotype, up to 15% of the lymphocytes double stain for both allotypes (14). It was also found that the presence of molecules of two allotypes on single cells is probably due to the binding of serum Ig to the lymphocyte membranes and that molecules of only one allotype return after cells are stripped of their membrane Ig with pronase. However, the cell transfer studies reported here were done before these observations were made. Consequently, some of the fractions purified for lymphocytes bearing b5 or b9 markers contained varying numbers of double-stained cells.

When highly purified populations of cells selected to bear a particular allotype

TABLE I

Effect of Membrane Staining on Numbers of b5 and b9 CSC in Recipients' Spleens after Transfer of Unseparated b5b9 Peyer's Patch Cells

Recipient no.	Inoculum		Donor CSC* in recipients' spleens	
	no. cells $\times 10^{-6}$	Membrane stain	no. CSC‡	% b5 (of total CSC)§
1	10	R-anti-b9 + F-anti-b5	2460	67
2	10	R-anti-b9 + F-anti-b5	1223	58
3	5	R-anti-b9 + F-anti-b5	4352	62
4	5	R-anti-b9 + F-anti-b5	1002	62
5	2	R-anti-b9 + F-anti-b5	1250	61
6	2	R-anti-b9 + F-anti-b5	995	74
7	10	None	615	63
8	5	None	430	50
9	2	None	934	69
10	2	None	4553	67

* Cytoplasmic Ig-stained cells.

‡ Total b5 and b9 CSC counted in one-half of cytocentrifuge pellet, containing $\leq 10^5$ nucleated spleen cells. Means of counts on duplicate slides (15% average difference between duplicate slides).

§ Means of determinations on duplicate slides (6% average difference between duplicate slides).

on their membranes were transferred into irradiated recipients, they gave rise predominantly to CSC producing Ig of the corresponding allotype (Fig. 2). Enrichments up to 100% for b5 CSC and 96% for b9 CSC were obtained in recipients of FACS fractions containing predominantly b5- or b9-bearing lymphocytes, respectively. In contrast, the transfer of unseparated Peyer's Patch lymphocytes into a total of nine recipients (including experiments not represented in Fig. 2) yielded an average of 69% b5 and 31% b9 CSC. In another experiment not depicted in Fig. 2, peripheral blood lymphocytes from a b^5/b^9 rabbit were fractionated into b5+ and b5− populations. The b5− cells, after transfer, gave rise to CSC which were 93 and 99% b9, respectively, in two recipients. The b5+ fraction was not of sufficient purity to use. It was also

found that the transfer of cells which had been stripped of membrane Ig with pronase and then cultured overnight before separation into b9+ and b5+ fractions yielded predominantly b9 and b5 CSC, respectively, in recipients in one experiment. Thus, in the transfer of highly purified cell populations, the

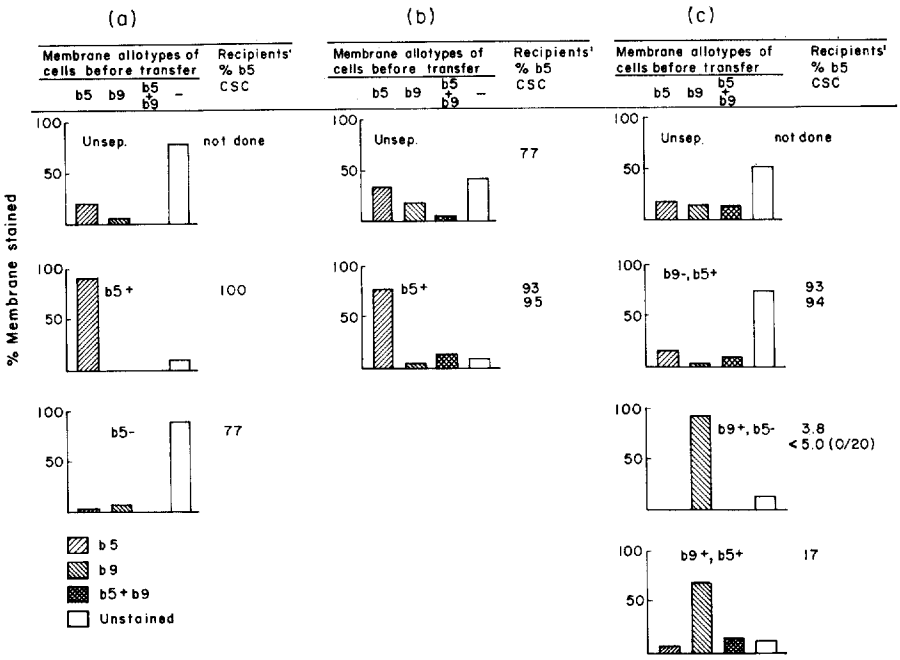

FIG. 2. b5 and b9 CSC in spleens of recipients of FACS-separated Peyer's Patch lymphocytes. The membrane-staining analysis and percent b5 CSC of total b5 plus b9 CSC in recipients' spleens 7 days after transfer are presented for three experiments. The cells passed through the FACS had not been previously treated with pronase in these experiments. (*a*) Cells were stained with F-anti-b5 and separated into b5+ and b5− fractions. The proportion of b9-bearing cells in the two fractions was estimated from the proportion of b5-negative cells in the unseparated population which was stained by R-anti-b9. The two recipients received 8×10^6 b5+ cells and 21×10^6 b5− cells, respectively. (*b*) The separation was performed as in *a*. The proportion of b5-bearing cells which also had b9 on their membranes was estimated from the proportion of b5-bearing cells in the unseparated population which double stained with the R-anti-b9 reagent. All three recipients received 10×10^6 cells. (*c*) Cells were stained with R-anti-b9 and separated into b9+ and b9− fractions, both of which were then stained with F-anti-b5 and separated for b5+ and b5− cells. The proportions of b5, b9 and double membrane-stained cells in each fraction was determined directly by microscopy after staining with the two reagents. 1.6×10^6 b9−, b5+ cells, 0.5×10^6 b9+, b5− cells, and 2.7×10^6 b9+, b5+ cells were transferred into the indicated number of recipients.

allotype of the lymphocytes' membrane Ig seems to be the same as the allotype of the Ig made by their CSC progeny in recipients' spleens.

When low numbers of cells, i.e., less than 10^6, were transferred, as was the case in some experiments in which the cells had been treated with pronase and

then cultured overnight, some recipients showed proportions of b5 and b9 CSC that did not correlate well with proportions of lymphocytes with membrane b5 and b9 markers. For example, a number of recipients of unseparated cells had only b5 CSC in their spleens, perhaps due to very low numbers of effective precursors lodging in the spleens, allowing the expansion of one or very few clones of cells. Because of this problem, encountered when trying to analyze the potential of small numbers of lymphocytes by allogeneic transfer, another system was developed for assaying the differentiative potential of separated lymphocytes.

Effect of Mitogens and Membrane Staining on Levels of CSC in Cultures of Peyer's Patch Lymphocytes.—The inclusion of the plant lectins PHA and PWM in microcultures of Peyer's Patch lymphocytes stimulates the appearance of CSC. Fig. 3 shows that the increase in total numbers of CSC recovered from cultures containing mitogen is due to elevations of both the viable cell recovery and the proportion of total cells which are cytoplasmic stained for Ig. The doses of mitogen used (0.4 μl/ml PHA and 25 μl/ml PWM) had been found to give maximal yields of CSC. However, the degree of stimulation of CSC levels by PHA and PWM varies from animal to animal, as does the time required for the peak CSC response to occur (Table II).

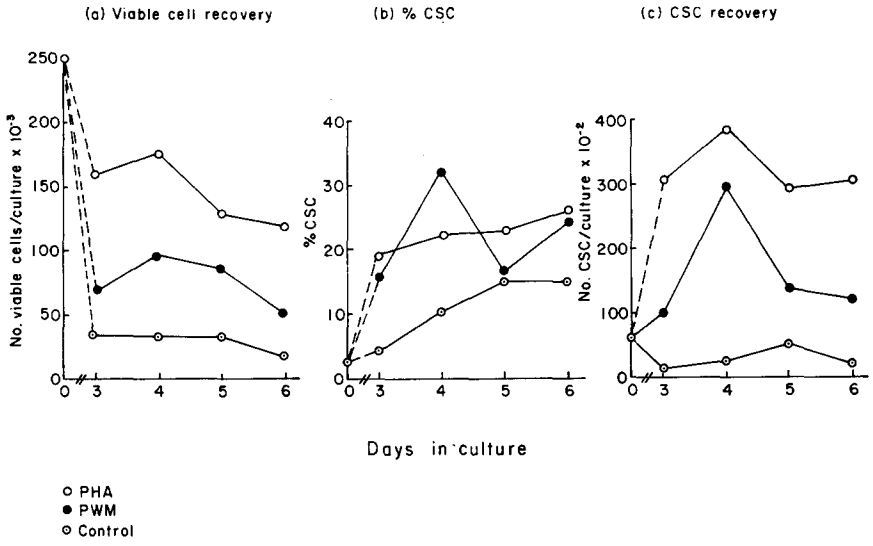

FIG. 3. Effect of mitogens on levels of viable cells and CSC in cultures of Peyer's Patch lymphocytes. Peyer's Patch lymphocytes from a b^4/b^4 rabbit were stripped of membrane Ig with pronase, cultured overnight to allow reappearance of membrane Ig, and then put into microcultures without mitogen (control) or with either 0.4 μl/ml PHA or 25 μl/ml PWM. On the day indicated, four cultures were pooled per group, and viable cell recoveries were determined. Cytocentrifuge preparations were stained with F-anti-b4 (14) and b4 CSC were counted.

It was necessary to determine whether prior membrane staining affects the recovery of b5 and b9 CSC from culture. Peyer's Patch lymphocytes from b^5/b^9 rabbits were stripped with pronase and allowed to regenerate their membrane Ig during overnight culture, eliminating exogenously bound Ig. Cells stained with both R-anti-b9 and F-anti-b5, as well as cells left unstained, were cultured with or without mitogen (Table III). The results indicate that prior staining does not consistently alter CSC yield, nor does either of the fluorescent antibody reagents selectively affect the number of CSC of a given allotype. Thus

TABLE II

Effect of Mitogen on Total and Cytoplasmic Ig-Stained Cell Recovery after Culture

Exp. no.	Mitogen*	no. viable cells/culture $\times 10^{-3}$‡		% CSC		no. CSC/culture $\times 10^{-2}$	
		Day 4	Day 5	Day 4	Day 5	Day 4	Day 5
1	—	32	35	10	15	32	52
	PWM	92	52	32	16	295	135
	PHA	175	128	22	23	385	292
2	—	73	33	4.0	13	29	43
	PWM	107	67	8.0	18	86	121
	PHA	ND§	31	13	11	ND	34
3	—	50	29	9.0	10	45	29
	PHA	125	119	8.4	7.6	105	90
4	—	78	26	13	12	101	31
	PWM	90	96	17	23	153	221
5	—	34, 34	26, 32	4.9, 3.4	3.8, 6.8	17, 12	10, 22
	PWM	54, 40	60, 64	8.2, ND	4.0, 4.5	33, ND	27, 26

Four cultures were pooled for each determination, except in Exp. 5 in which day 4 and day 5 determinations were on two individual cultures and two pools of two cultures, respectively.

* 0.4 μl/ml PHA; 25 μl/ml PWM.

‡ Cultures initially contained 2.5×10^5 cells (day 0).

§ ND, not done.

TABLE III

Effect of Membrane Staining and Mitogen on Levels of b5 and b9 Cytoplasmic Ig-Stained Cells after 4 Days in Culture

Cultures			no. CSC/culture $\times 10^{-2}$				% b5 (of total CSC)			
Cells		Mitogen*	Exp. 3	Exp. 5	Exp. 6	Exp. 7	Exp. 3	Exp. 5	Exp. 6	Exp. 7
Unstained		−	45	16	33	17	67	72	78	62
		+	105	40	73	47	67	70	78	64
Membrane stained‡		−	125	14	36	33	68	70	74	56
		+	57	33	97	55	66	67	74	59

* Exp. 3, 0.4 μl/ml PHA; Exp. 5–7, 25 μl/ml PWM.

‡ Peyer's Patch lymphocytes were membrane stained with both R-anti-b9 and F-anti-b5 before culture.

the binding of fluorescent antiallotype antibodies to the surfaces of lymphocytes separated using the FACS should not alter their ability to differentiate into CSC in culture.

Microculture of Lymphocytes Separated According to Membrane Allotype.— Some of the cells in the experiments just described (Table III) were passed through the FACS and separated according to membrane allotype, after which they were cultured in the presence of mitogen. After 4 days in culture the cells were harvested, quadruplicate cultures were pooled, and the numbers of b5 and b9 CSC per culture were determined. Fig. 4 shows that the b9+ fraction (90% b9 bearing) and the b9−, b5+ fraction (91% b5 bearing) gave rise to CSC which were 91% b9 and 99% b5, respectively. Thus there is a close concordance between the allotype of a lymphocyte's membrane Ig and the allotype of the Ig synthesized by the CSC descendants of that lymphocyte. In addition, Fig. 4 shows that the lymphocytes without membrane Ig generate

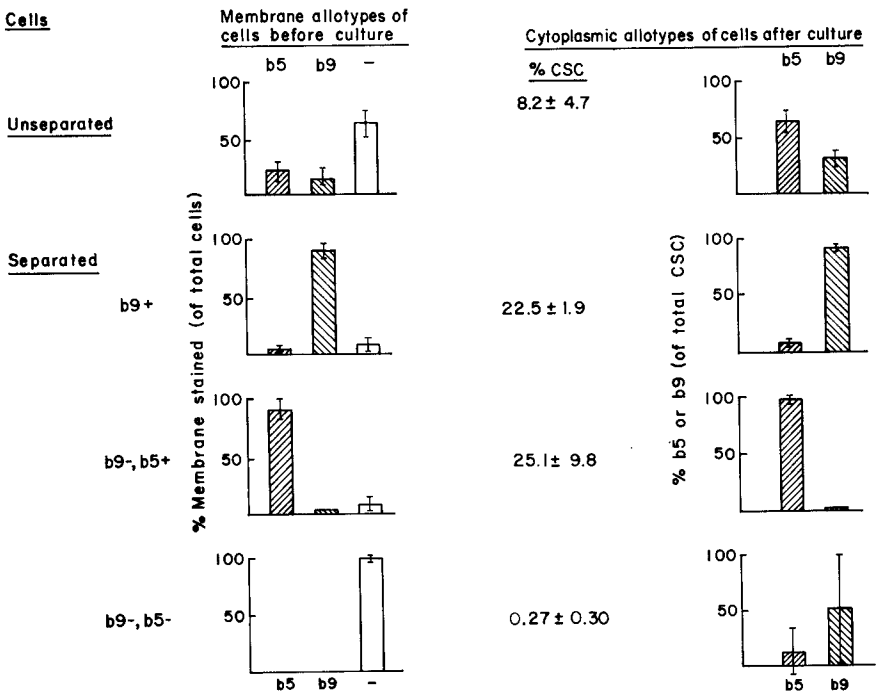

FIG. 4. Microculture of lymphocytes separated according to membrane allotype. Peyer's Patch lymphocytes, previously stripped with pronase and cultured overnight to regenerate membrane Ig, were stained with R-anti-b9 and separated into b9+ and b9− fractions. The latter were then stained with F-anti-b5 and separated into b9−, b5+ and b9−, b5− fractions. Unseparated and separated cells were put into microculture in the presence of mitogen. 4 days later cells were harvested, and pools of four cultures were counted and stained for CSC. The data are presented as the means ±1 standard deviation for four experiments (3, 5–7, cf. Table III).

very few CSC in culture, usually only about 1% of the number found in cultures of Ig-bearing cells.

Because some of the CSC found after 4 days in culture might have been the same CSC which were present in the lymphocyte fractions initially put into culture, the numbers of b5 and b9 CSC at the start and conclusion of the culturing period were compared (Table IV). In cultures of some fractions the absolute number of CSC was not significantly greater than the number present at the start of culture. Although most cultures initially contained less than 2% CSC, the low overall viable cell recoveries in some cultures after 4 days resulted in a low recovery of CSC (Table V). In both Exp. 6 and 7 the addition of equal numbers of Ig-negative b9−, b5− cells to the b9+ cells increased both the overall viable cell recovery and the number of b9 CSC per culture (Table IV),

TABLE IV

*Changes in Numbers of Cytoplasmic Ig-Stained Cells during culture of Cell Sorter Fractions**

Cells	no. CSC/culture ($\times 10^{-2}$)			
	Exp. no. 6 Before culture/after culture		Exp. no. 7 Before culture/after culture	
	b5 CSC	b9 CSC	b5 CSC	b9 CSC
Unseparated	19/70	6.2/25	42/32	20/23
b9+	0/3.9	40/45	0/3.1	48/41
b9−, b5+	18/94	0/0	33/46	0/0
b9−, b5−	28/0	4.0/0.8	2.5/2.5	2.5/3.5
Mixtures				
50% b9+/50% b9−, b5−	14/4.2	22/43	1.2/5.4	25/103
50% b9−, b5+/50% b9−, b5−	23/54	2.0/0.6	ND‡	ND

* Cells were cultured for four days in the presence of 25 μl/ml PWM.

‡ ND, not done.

especially since only 50% as many b9+ precursor cells were present compared to cultures of b9+ cells alone. That all of the CSC were derived from the b9+ cells is shown by the continued very low proportion of b5 CSC after culture; if the b-negative (b9−, b5−) cells had contributed significant numbers of CSC, some of these should have been b5. In contrast, the addition of equal numbers of Ig-negative cells to the b5-bearing cells produced only a small enhancement of the CSC response compared to numbers of b5 CSC in cultures of b9−, b5+ cells alone, but even in cultures of b9−, b5+ cells alone there was a fivefold increase in the number of b5 CSC during the 4 days of culture. These cells already may have been given a maximal response. Thus both the b9-bearing and b5-bearing lymphoid cell populations are capable of generating new CSC in vitro, and these CSC synthesize the same allotype as the membrane Ig of their precursor lymphocytes.

TABLE V

*Levels of b5 and b9 Cytoplasmic Ig-Stained Cells after Culture of Cell Sorter Fractions**

Exp. no.	Cells	no. viable cells/culture $\times 10^{-3}$	% CSC	no. CSC/culture $\times 10^{-2}$	% (of total CSC)	
					b5	b9
6	Unseparated	66	15	97	74	26
	b9+	24	20	49	8	92
	b9−, b5+	36	26	94	100	0
	b9−, b5−	42	0.2	0.77	5	95
	50% b9+/50% b9−, b5−	52	9.0	47	9	91
	50% b9−, b5+/50% b9−, b5−	74	7.4	55	99	1
7	Unseparated	65	8.5	55	59	41
	b9+	18	24	44	7	93
	b9−, b5+	12	38	46	100	0
	b9−, b5−	107	0.6	6	41	59
	50% b9+/50% b9−, b5−	82	13	108	5	95

* Cells were cultured for 4 days in the presence of 25 μl/ml PWM.

DISCUSSION

The results of the present analyses of cells from heterozygous rabbits indicate that the B lymphocytes and plasma cells which comprise a clone are committed to the synthesis of the same allotype of Ig. The ability to isolate with the FACS nearly pure populations of lymphocytes bearing molecules of one allotype on their membranes has enabled us to demonstrate directly that the allotype of a lymphocyte's membrane Ig is the same as that synthesized by its plasma cell descendants.

Both PHA and PWM, used in these studies to enhance the CSC response, are known to stimulate the proliferation and maturation of lymphocytes. Soluble PHA predominantly stimulates the proliferation of T cells among mouse lymphocytes (27, 28), although in the presence of T cells some mitogenic effects on B cells have also been noted (29, 30). PHA has been found by a number of laboratories to stimulate the maturation of B lymphocytes to antibody-forming cells, although this point is still controversial. Hirschhorn et al. initially described increased Ig synthesis by human peripheral blood cells cultured with PHA (31), but this effect could perhaps be ascribed to a general stimulation of overall protein synthesis rather than of the maturation of lymphocytes to plasma cells (32, 33). More recently, however, Kreth and Herzenberg have shown that PHA stimulates the numbers of cells with cytoplasmic Ig in cultures of B lymphocytes from human peripheral blood.[3] Submitogenic

[3] Kreth, W. and L. A. Herzenberg. 1973. Fluorescence-activated cell sorting of human T and B lymphocytes. I. Direct evidence that lymphocytes with a high density of membrane-bound immunoglobulin are precursors of plasmacytes. *Cell Immunol.* In press.

doses of PHA also enhance the in vitro hemolytic plaque-forming response of mouse cells to sheep erythrocytes (34).

PWM is mitogenic for both B and T cells from the mouse and can stimulate B cells in the absence of T cells (27). PWM also has a profound effect on the maturation of B lymphocytes into plasma cells. Ultrastructural studies (35) of pokeweed-stimulated human peripheral blood lymphocytes and of mouse spleen cells (36) have revealed that a large proportion of cells have the morphological characteristics of plasma cells. An increase in Ig synthesis and secretion also has been demonstrated in PWM-containing cultures of human[3] and mouse (37) cells, apparently due to a nonspecific stimulation of the differentiation of large numbers of B lymphocytes into plasma cells.

Less is known about the effects of these mitogens on subpopulations of rabbit lymphocytes, but the results of Elfenbein et al. (30) suggest that both B- and T-cell analogues in rabbit peripheral blood can be induced to proliferate by PHA and PWM. We have reported here that the addition of either of these mitogens to cultures of Peyer's Patch lymphocytes stimulates the CSC response by increasing both the number of viable cells per culture and the proportion of these which are CSC. Purified populations of Ig-bearing cells in this system are capable of giving rise to CSC in the absence of added Ig-negative cells; however, the yields of CSC in cultures of Ig-bearing cells could be improved by the addition of negative cells. Whether this enhancement was the result of a synergistic interaction between populations equivalent to mouse B and T cells (not yet well defined for the rabbit), or whether the culture conditions simply were more conducive to the differentiation of lymphocytes to CSC, is not clear. The viable cell recovery generally was better in cultures of unseparated or Ig-negative cells than in cultures of Ig-bearing cells. Janossy and Greaves (27) have in fact shown that cell density is critical in obtaining the maximum proliferative response of mouse spleen cells to PHA or PWM. This variable, plus the added complications that (*a*) the viable cell recovery and the number of CSC per culture are not always maximal on the same day (Table II), and (*b*) the Ig-positive and Ig-negative populations can differ in when they reach their peak numbers of viable cells (P. Jones, unpublished observations) interfere with determining most comprehensively the capacities of these cells to proliferate, differentiate, and mature. A more detailed analysis of the cellular basis for the stimulation of the CSC response by mitogen will be presented in a subsequent paper.[4]

It is possible that some of the CSC present after 4 days in culture were generated by division of CSC already present before culture, but it is unlikely that the majority of them were derived from such previously existing CSC. The isotype of the Ig synthesized by the majority of CSC changes during the 4 days in culture, so that the absolute increase in CSC of the class becoming numerically dominant is even greater than the zero- to sixfold increases in total CSC reported in this paper. In addition, the ability of fewer than 5×10^5 transferred Peyer's Patch lymphocytes to successfully repopulate the spleens of the irradiated recipients as shown here, in addition to seeding the gut lamina propria (25), indicates that the lymphocytes certainly have the capacity to give rise to plasma cells. Until 5 or 6 days after transfer few CSC of donor allotype are present in recipients' tissues, but then an explosive in-

[4] Jones, P. P., S. W. Craig, J. J. Cebra, and L. A. Herzenberg. 1974. Restriction of gene expression in B lymphocytes and their progeny. II. Commitment to immunoglobulin heavy chain isotype. Manuscript in preparation.

crease in CSC occurs, probably after the final step in the maturation of the donor lymphocytes. The daughter CSC, in either recipients' spleens or in microculture, contain Ig of the same allotype as the membrane Ig of their lymphocyte precursors.

The phenomenon of allelic exclusion, in which Ig molecules of only one allotype are synthesized by each lymphoid cell from a heterozygous animal at any given time, is a unique feature of immunocytes. No other known autosomal loci exhibit this phenotypic restriction. The one other example of a limitation of gene expression to one of the two homologous chromosomes in mammalian cells is the inactivation of one of the X chromosomes in females by heterochromatization (38). In this phenomenon the inactivation takes place early in development, and the decision as to which chromosome is inactivated is immutable during the lifetime of the clone (39).

It is generally accepted that the great majority of plasma cells synthesize only one molecular species of Ig (7), but it has been more difficult to show that all the members of a clone of lymphoid cells are restricted to the synthesis of one Ig species. Although the exquisite specificity of the antibody response predicts that all the cells in a clone produce Ig with the same specificity, as outlined by Burnet's clonal selection theory (40), a number of studies suggest that there can be a switch from one kind of heavy chain to another during the maturation of a clone (8, 9). Such a switch would not be incompatible with the need for maintaining synthesis of the same variable region, if, indeed, separate genes control the synthesis of the variable (V) and constant (C) regions of the same chain (41). Genetic loci controlling the expression of the different heavy-chain constant regions appear to be linked in the mouse (42, 43), man (44), and rabbit; in the rabbit the loci controlling the C_γ (45), C_μ (46), and C_α (47) allotypes are linked also to the *a* locus, which controls specificities in the V regions of all heavy chains. V and C region linkage is also suggested by the association between loci determining C_H allotypes and those controlling some idiotypic specificities in the mouse (48–50). Similarly, the association between b-allotype-related amino acids in the constant region of κ-light chains with particular sets of residues in the variable region of the same chain suggests linkage of V_L and C_L in the rabbit (51, 52). Thus, perhaps by successive translocational events (53), a single V region could be associated with different C regions during the lifetime of a clone, one chromosome coding for all component parts of the Ig chain.

With these observations implying linkage of V and C regions at the genetic and product levels in mind, it is more difficult to conceive of how the specificity of the antibody produced by a clone could be conserved if changes could occur in the allotype of the molecule. If the determination in a stem cell of which V region DNA eventually will be transcribed is random, as postulated by the clonal selection theory, then that choice might be different on each of the two homologous chromosomes in the cell. Thus, if the products of both chromosomes could be expressed within a clone, the stimulation of a lymphocyte bearing receptors of one specificity by the appropriate antigen might lead to synthesis of two different antibodies. Such degeneracy would reduce both the specificity and the efficiency of the immune response. Hence, the restriction of each lymphoid cell clone to the expression of Ig genes on a single chromosome, as revealed by the commitment to allotype, may guarantee the maximally effective humoral immune response.

SUMMARY

Lymphocytes from b^5/b^9 rabbits were stained in suspension with fluorescent antiallotype antibody reagents to selectively label with fluorescent molecules those cells bearing membrane immunoglobulin (Ig) of the b5 or b9 allotype. After staining, the cells were separated by the fluorescence-activated cell sorter into populations markedly enriched in cells bearing b5 or b9 membrane Ig or totally depleted of cells with detectable membrane Ig. The potential of these separated cells to give rise to Ig-synthesizing plasma cells either in vivo after transfer into irradiated recipients or in vitro during culture in the presence of phytohemagglutinin or pokeweed mitogen was assessed by immunofluorescence. The relative proportion of b5 and b9 cytoplasmic Ig-stained cells (CSC) arising from the separated cells was determined to test directly whether B lymphocytes and their progeny are committed to the synthesis of Ig of one allotype.

It was found that b5- and b9-bearing cells gave rise almost exclusively to b5- and b9-producing plasma cells, respectively, in both the in vivo and in vitro assay systems. Most of these CSC were probably not derived from previously existing CSC but arose as the result of the differentiation of lymphocytes with membrane Ig. When cell populations totally depleted of Ig-bearing lymphocytes were cultured, very few CSC were found, indicating that the majority of immediate precursors of CSC have membrane Ig. These results suggest that individual B cell clones are phenotypically restricted to the expression of immunoglobulin genes on one chromosome; the significance of this clonal allelic exclusion is discussed.

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