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IMMUNOGLOBULIN (Ig) ALLOTYPE MARKERS ON RABBIT LYMPHOCYTES: SEPARATION OF CELLS BEARING DIFFERENT ALLOTYPES AND DEMONSTRATION OF THE BINDING OF Ig TO LYMPHOID CELL MEMBRANES[1]

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Lymphocytes from peripheral blood and Peyer's patches of b^5b^9 and b^4b^5 rabbits were membrane stained with rhodamine- and fluorescein-labeled anti-allotype reagents. Up to 63% of peripheral blood lymphocytes and 15% of Peyer's patch lymphocytes were found to double stain for both allotypes on their membranes. With a fluorescence-activated electronic cell sorter, cell populations were fractionated according to membrane allotype. A double pass separation procedure after staining yielded four distinct cell populations: one each of cells single-stained for one of the two allotypes, one containing cells double-stained for both allotypes, and one of unstained cells. Properties of the cells bearing two allotypes were examined. Cells put into culture after they were stripped of their membrane immunoglobulin (Ig) with Pronase regenerated their membrane Ig; however, the proportion of cells bearing both allotypes was greatly reduced. This suggested that the lymphocytes were restricted to the synthesis of membrane Ig molecules of a single allotype at a given time. It was found that a high proportion of rabbit lymphocytes could bind exogenous Ig from serum in vitro and probably also in vivo, explaining the presence of molecules of both allotypes on individual cells.

The presence of immunoglobulin (Ig) determinants on the surfaces of lymphocytes has now been well documented for many species. A variety of staining techniques utilizing anti-Ig antibodies, including immunofluorescence (1, 2), autoradiography (3, 4), immunocytoadhesion (5, 6), and electron microscopy (7, 8) has permitted direct visualization of cells bearing Ig molecules. Similar methods have been used to show the binding of antigen to lymphocytes (2, 9, 10).

Evidence suggesting that this membrane Ig functions as the receptor for antigen has been provided by experiments utilizing anti-Ig antibodies to block immune responses *in vivo* (11–13) and *in vitro* (14, 15). However, since these and other (16, 17) experiments have been based primarily on the inhibition or depletion of the response to antigen, it is possible that the observed effects of anti-Ig reagents were indirect, being on molecules or cells not directly involved in antigen binding. The ability of anti-Ig antibodies to block binding of radiolabeled antigen by lymphocytes, thus preventing "suicide" of specific antigen-binding cells (12), is a more direct indication that the receptor is Ig. Further evidence for the role of membrane Ig as the receptor for antigen has been provided by recent experiments with enriched populations of antigen-binding cells to generate an antibody response (18, 19). Similarly, the ability to obtain populations of lymphocytes, purified according to their membrane Ig, should facilitate determining whether or not the particular

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polymorphic form of Ig displayed by a lymphocyte on its membrane is necessarily identical to the molecule that its daughter lymphocytes and plasma cells will synthesize at some time in the future.

Presented here are studies of the functional significance of membrane Ig on rabbit lymphocytes, with *b* locus (κ-chain) allotypes as markers. Fluorescent antibody staining revealed that some lymphocytes from rabbits heterozygous at the *b* locus have molecules of both allotypes on their membranes; the origin and significance of this double-staining were explored. In this paper we also report the use of a fluorescence-activated cell sorter (19, 20) to separate lymphocytes from heterozygous rabbits into fractions purified for cells bearing molecules of one or the other allotype.

MATERIALS AND METHODS

Animals. Rabbits homozygous for the b4 light chain allotype were obtained from commercial breeders (Bar F Rabbitry, Perry Hall, Md.; B and H Rabbitry, Rockville, Md.). Heterozygous b^4b^5 rabbits were either obtained from these sources or were raised in our own animal facilities. Some of the b^5b^5, b^9b^9, and b^5b^9 rabbits used in these studies were kindly donated by Drs. R. G. Mage and C. W. Todd; others were raised in our facilities. Goats were maintained at the Johns Hopkins University animal farm.

Anti-allotype reagents. For use as immunogen, IgG prepared as previously described (21) was insolubilized with bis-diazobenzidine (BDB)[3] (22). Rabbit anti-b5 and anti-b9 antisera were raised by the injection of 5 mg of BDB-b5 or -b9 IgG in complete Freund's adjuvant (CFA, Difco Laboratories, Detroit, Mich.) intradermally into the flanks and footpads of b^4b^4 rabbits of the same *a* locus allotype as the donor of the immunogen. Three weeks later animals were given an additional 3 to 5 mg of BDB-IgG in incomplete Freund's adjuvant (IFA, Difco Laboratories) into the flanks; rabbits were bled 2 weeks later. If the antibody titer was judged to be too low, rabbits were restimulated with a series of three daily i.v. injections of 1 mg of soluble IgG and then were bled 1 week later.

Goat anti-b4 was raised by intradermal and intramuscular injection in CFA of 10 mg light chain obtained from b4 IgG by the method of Fleischman *et al.* (23). The IgG fractions of this antiserum were absorbed sequentially with b5 IgG and light chain prepared from b5b9 IgG, both coupled to Sepharose-2B. Goat anti-b5 was prepared similarly: IgG from anti-b5 light chain antiserum was absorbed with b4 IgG, b9 IgG, and b4 light chain immunoadsorbents. Because this goat antiserum also contained antibodies which reacted with rabbit lymphocytes, it was then absorbed with pooled spleen, thymus, and appendix cells from b^4b^4 rabbits. All anti-allotype reagents reacted in Ouchterlony gel diffusion only with Ig of the same *b* locus allotype as the immunogen.

Rhodamine (R) and fluorescein (F) conjugates of IgG fractions of the anti-allotype antisera were prepared according to the method of Cebra and Goldstein (21), sterilized by Millipore filtration, and stored at 4°C.

Preparation of cells. Peyer's patch cells were obtained by teasing apart the tissues at 4°C in Eagle's minimal essential medium (MEM, Grand Island Biological Co., Grand Island, N. Y.) or Dulbecco's phosphate-buffered saline (PBS)[4] containing 5% fetal calf serum (FCS) and 1% antibiotic-antimycotic solution (AA, 10,000 units/ml penicillin, 10,000 μg/ml streptomycin, 25 μg/ml Fungizone) (both from Grand Island Biological Co.). The cells were passed through a stainless steel mesh and several milliliters of packed washed glass wool before being washed three times.

For preparation of peripheral blood lymphocytes, heparinized blood, collected from the marginal ear vein or by cardiac puncture, was mixed with an equal volume of 3% gelatin in Ringer's solution. After allowing red cells to sediment at 37°C for 30 min, the supernatant was centrifuged and the cells were washed once at 25 or 37°C with medium (MEM or PBS containing 5% FCS and 1% AA) plus 5 units/ml heparin. Erythrocytes were lysed by incubation for 3 to 10 min in 0.83% ammonium chloride

[3] Abbreviations used in this paper: BDB, bis-diazobenzidine; CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant; F, fluorescein; R, rhodamine; MEM, minimum essential medium, PBS, phosphate-buffered saline; FCS, fetal calf serum; AA, antibiotic-antimycotic solution; OA, ovalbumin; B, bone marrow-derived.

[4] In experiments using the fluorescence-activated cell sorter, PBS was used instead of MEM; no detectable difference in membrane staining results was noted with the two different media.

either in Gey's solution minus sodium chloride or 0.01 M phosphate buffer, pH 7.5. The cells were passed through a glass wool column and washed again before being counted in a hemocytometer. Peyer's patch cells were 80 to 95% viable as judged by trypan blue exclusion; peripheral blood cells were 95% viable. Giemsa staining indicated that cell preparations from both tissues routinely contained 95% lymphocytes.

Pronase stripping. Cells were suspended at 1 to 3 $\times$ 10^7 cells/ml in MEM or Hanks' balanced salt solution (Grand Island Biological Co.), with or without 0.25% Pronase (Pronase, Calbiochem, San Diego, Calif., or *Streptomyces griseus* Protease Type VI, Sigma Chemical Co., St. Louis, Mo.). After incubation at 37°C for 30 min, the treated cells were centrifuged through FCS and were washed twice in medium at 4°C.

Cell culture. The media used for cell preparation and for culture were essentially those of Mishell and Dutton (24), as modified for rabbit cells by Henry *et al.* to include asparagine, antibiotics, and extra vitamins (25). The FCS used for culture was obtained from the Colorado Serum Co., Denver, Colo. Cells were cultured at 10^7 cells/ml, 1 ml/culture, in 30-ml disposable screw-cap culture flasks (Falcon Plastics, Oxnard, Calif.), which were gassed and supplemented daily with a nutritional cocktail (25). In a few experiments involving overnight culturing only, cells were cultured in MEM + 10% FCS at 2 $\times$ 10^6 cells/ml, 2 ml/culture, in 12 $\times$ 75-mm disposable plastic tubes (Falcon Plastics) in a 5% CO_2 in air incubator.

Membrane staining. For staining, 0.5 to 2.0 $\times$ 10^7 cells were centrifuged in siliconized 3-ml conical glass tubes, resuspended in 0.1 ml of a fluorescent anti-allotype reagent, and incubated 30 min at 4°C. For staining fewer lymphocytes (5 $\times$ 10^4 to 5 $\times$ 10^6), chicken erythrocytes previously fixed with 0.1% glutaraldehyde were added as carrier cells. After staining, cells were washed with medium and then centrifuged through FCS. If a second staining step was required, cells were again incubated in a fluorescent antibody reagent and washed as before. Cells were then resuspended in a drop of FCS, smeared on microscope slides, fixed in 95% ethanol, and mounted in a 9:1 mixture of glycerol and PBS under a cover slip for observation. In some cases cells were viewed live in suspension under the microscope; there was no significant difference in the intensity of fluorescence, but fixed preparations were more satisfactory since they lasted longer and permitted examination of cell morphology. Only cells with the morphologic appearance of lymphoid cells were counted. Routinely 100 to 300 lymphocytes were scored, except for the negative control stainings when at least 1000 cells were inspected.

Combinations of reagents to be used for double staining were selected so that there would be no reaction between them. Thus F-labeled anti-b5 and R-labeled anti-b9 (both raised in b^4b^4 rabbits) were used to stain cells from b^5b^9 rabbits, and F-goat anti-b4 and R-goat anti-b5 were used for b4b5 cells. Reagent concentrations giving optimal staining were determined and used for each reagent, generally 1 to 3 mg/ml. The goat anti-b4 and anti-b5 reagents were mixed together for staining, but the rabbit anti-b5 and anti-b9 were used sequentially because of the concentrations available. The order of staining did not affect results.

Fluorescence microscopy. Two fluorescence microscopes were used during these studies, one at Johns Hopkins University and one at Stanford University. The filter combinations used for detecting R and F fluorescence have been described in detail for the Johns Hopkins microscope (26), and also for the Stanford microscope (19) to which was added a Zeiss narrow-band pass interference filter PiL, λ_{max} = 546 nm $\pm$ 2 nm, 32 mm diameter for exciting R and a double layer of Kodak Wratten gelatin filter No. 23A for a corresponding barrier filter.

Cell separations. A block diagram of the fluorescence-activated cell sorter used in these studies is given in Figure 1.

Membrane stained Peyer's patch or peripheral blood lymphocytes were suspended in PBS + 5% FCS and passed through the cell sorter, usually at a rate of 1 to 5 $\times$ 10^7 cells/hr. Both the fluorescence-deflected and undeflected fractions were concentrated and stained with a second, contrastingly-labeled fluorescent antibody reagent before being re-passed through the cell sorter.

RESULTS

Membrane staining for allotype. The proportions of peripheral blood and Peyer's patch lymphocytes from heterozygous b^5b^9 rabbits which membrane stained with the F-anti-b5

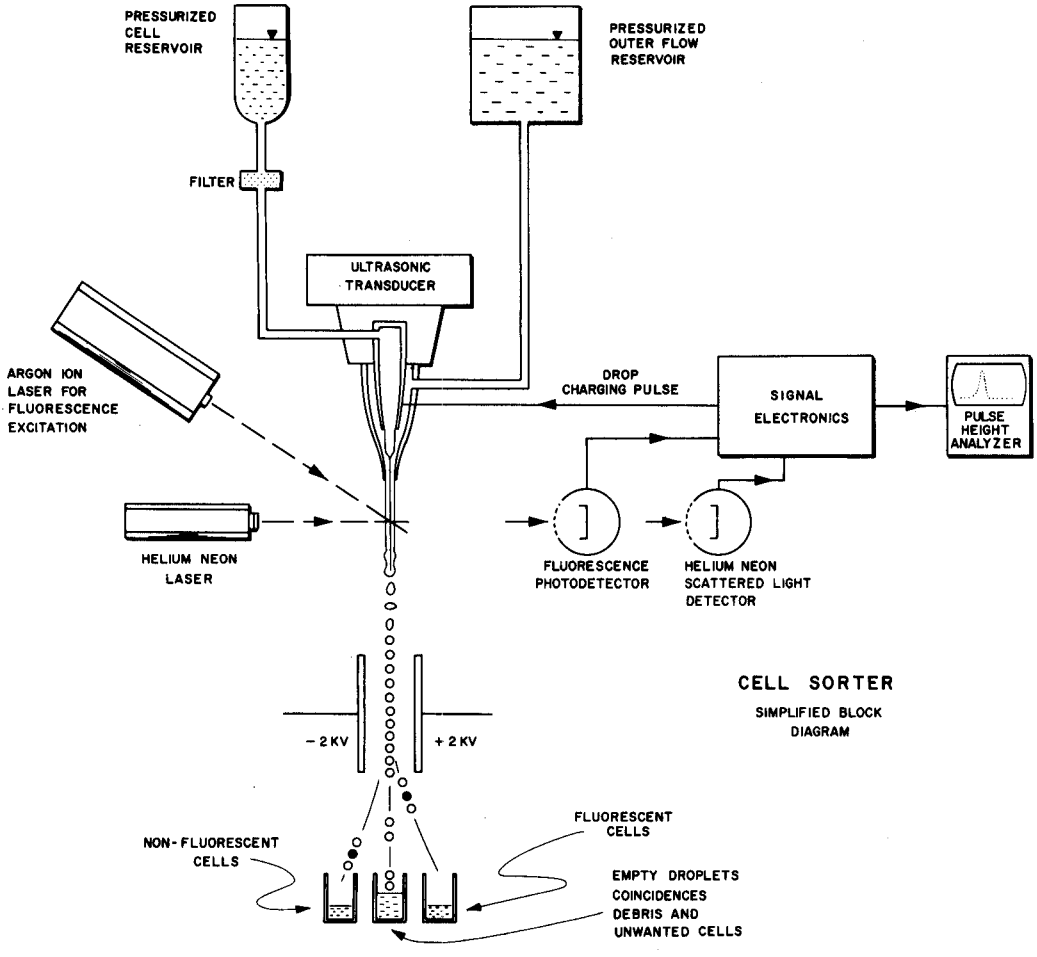

Figure 1. The present fluorescence-activated cell sorter enables separation of cells binding a detectable quantity of fluorochrome-conjugated protein (about 1×10^4 fluorescein or rhodamine residues/cell). Cells in liquid suspension are forced under pressure through a micro-nozzle that produces an effluent jet 50 μ in diameter. The architecture of this nozzle creates a coaxial flow system whereby cells injected along the center line are confined to about the central 12 μ of the effluent jet. The nozzle is vibrated axially at 4×10^4 cycles/sec, breaking the jet into 4×10^4 uniform droplets/sec. Immediately below the nozzle, before droplet formation occurs, the jet passes through a focused argon ion laser beam that excites fluorescence on those cells bearing fluorochrome-conjugated protein. Fluorescent light from such cells is imaged on a photomultiplier tube with darkfield optics and appropriate filters to exclude scattered exciting light. Also incident on the jet is light from a helium neon laser. Light scattered from this laser at small forward angles produces a signal proportional to the size of the cell. This light falls on a second photomultiplier and produces a signal that is used to verify that the fluorescent particle is of cellular dimensions. When this photomultiplier indicates presence of a cell, and at the same time a cell-derived voltage pulse from the fluorescence multiplier exceeds a preset limit, the effluent jet is given a positive electrical charge. When a cell is present, but there is little or no fluorescent signal, the jet is charged negatively. The charging signal is timed so that the detected cell will be trapped in a charged droplet formed during the charging interval. Droplets formed in the absence of a charging voltage will be uncharged. The stream, subsequent to droplet formation, passes through an electrostatic field, which causes deflection of positively charged droplets into one container. Uncharged droplets remain undeflected and collect in another container, while negatively charged droplets are deflected into a third container. The cells are maintained at 4°C throughout the separation except for a few seconds during actual deflection.

and R-anti-b9 reagents are presented in Table I. An average of 48% of Peyer's patch cells and 67% of peripheral blood cells stained for allotype markers. In both tissues there were considerable numbers of cells which double-stained for both b5 and b9 markers; the average fraction of cells with membrane Ig that had both b5 and b9 was 0.21 for Peyer's patch and 0.60 for peripheral blood cells. Table II summarizes the results of staining peripheral blood lymphocytes from b^4b^5 rabbits with F-anti-b4 and R-anti-b5. The total percentage of cells which stained for allotype, 62%, was similar to that for peripheral blood lymphocytes from b^5b^9 rabbits. The proportion of these which double-stained for both b4 and b5, 0.20, was lower.

The membrane stained cells exhibited the patched (spotted) distribution of fluorescence, typical of cells stained and kept at 4°C, whether observed live in suspension or on fixed smears. The size and density of fluorescent patches on individual cells covered a wide range, and the pattern was seemingly independent of cell size. Cells which were double-stained for two allotypes usually had more of one fluorescent anti-allotype than the other, and patches of the two anti-allotype stains often had different distributions on the cell surface.

Because of the relatively high proportions of cells staining for two allotypes, it was important to verify the specificity of the fluorescent anti-allotype reagents. Table III shows that each fluorescent anti-allotype reagent reacted only with cells from animals of the corresponding genotype. An additional control is given for the goat anti-allotype reagents, since results in our laboratory (S. Craig, unpublished observations) obtained with an indirect (sandwich) staining technique have shown that many goat sera contain antibodies which react with membrane components on rabbit lymphocytes. The goat anti-b4 and anti-b5 fluorescent reagents were absorbed reciprocally with b4 IgG and b5 IgG coupled to Sepharose and then were used to stain cells from rabbits 14 and 15. Each immunoadsorbent completely removed the staining ability of the anti-allotype reagent of corresponding specificity but did not diminish the activity of the reciprocal reagent. Thus, as judged by direct membrane staining, these reagents are specific for allotype determinants controlled by the *b* locus.

Cell separations. To obtain populations of cells bearing one or the other allotype marker on their membranes, lymphocytes from b^5b^9 rabbits were stained with fluorescent antibody reagents and passed through the fluorescence-activated cell sorter. Staining and separating cells for one allotype alone, however, was not sufficient to obtain a uniform cell population because of the high proportion of cells which carried both b5 and b9 Ig. Therefore, a double pass separation procedure was followed (Fig. 2), in which cells were first stained with one

TABLE I

Membrane staining of lymphocytes from b^5b^9 rabbits

Rabbit No.	Peyer's Patch (% Stained)				b5b9/Sum	Rabbit No.	Peripheral Blood (% Stained)				b5b9/Sum
	b5	b9	b5b9	Sum			b5	b9	b5b9	Sum	
1	26	13	5	44	0.11	7	21	16	26	63	0.41
2	34	19	6	59	0.10	8	27	12	17	56	0.30
3	22	5	8	35	0.23	9	7	1	41	49	0.84
4	25	20	12	57	0.21						
5[a]	18	15	14	47	0.30	5	5	9	63	77	0.82
						5[a]	8	15	63	86	0.73
6[b]	11	20	15	46	0.33	6	10	9	61	80	0.76
						6[b]	19	12	45	76	0.59
				48 ± 3.6[c]	0.21 ± .09					67 ± 6.9	0.60 ± .22

[a] Rabbit 5 peripheral blood experiments were done 4 days apart; Peyer's patch cells were obtained 3 days later.

[b] Both the second peripheral blood and the Peyer's patch experiments for rabbit 6 were done 9 days after the first peripheral blood experiment.

[c] Values are means ± 1 standard error.

fluorescent anti-allotype reagent and passed through the cell sorter with the optical conditions (exciting wavelength, barrier filters, and threshold voltage) optimal for that fluorochrome. The resulting two fractions, one enriched and one depleted for cells stained for that allotype, were then concentrated by centrifugation, stained with the contrastingly-labeled reagent specific for the second allotype, and repassed through the separator with optical conditions now optimal for that fluorochrome. This procedure resulted in the four final fractions indicated in Figure 2.

The results of three separations are presented in Figure 3. In experiment A cells were stained and separated first with F-anti-b5 and then with R-anti-b9. In experiments B and C the order of staining was reversed because conditions for separation of F-stained cells gave better discrimination between the two fluorochromes and hence were more appropriate for the second separation. In the three experiments the first separation produced enriched fractions ("b5+" or "b9+") containing, respectively, 91, 94, and 96% cells stained for the deflected allotype (sum of % single-stained for that allotype plus % double-stained), and depleted fractions ("b5−" or "b9−") contaminated with 11, 12, and 5% stained cells, respectively. Repassing these fractions through the cell sorter, deflecting for the second allotype, gave somewhat more variable results, but in general there was an enrichment for the expected class of stained cells. Some of the deviation from expected results might have occurred because of differences between the sensitivity of the eye

TABLE II

Membrane staining of peripheral blood lymphocytes from b^4b^5 rabbits

Rabbit No.	% Stained				b4b5/Sum
	b4	b5	b4b5	Sum	
10	36	10	10	56	0.18
11	25	9	12	46	0.26
12	41	6	7	54	0.13
13	53	3	16	72	0.23
14	52	11	12	75	0.16
15	50	5	16	71	0.23
				62 ± 0.6[a]	0.20 ± 0.05

[a] Values are means ±1 standard error.

TABLE III

Specificity of fluorescent anti-allotype reagents

Rabbit No.	Allotype	Reagents Used[a]				% Stained				
		F-goat anti-b4	R-goat anti-b5	F-rabbit anti-b5	R-rabbit anti-b9	b4	b5	b9	b4b5	b5b9
16	b^4b^4			+	+		0	0		0
17	b^4b^4	+	+			68	0		0	
18	b^5b^5			+	+		65	0		0
19	b^5b^5			+	+		80	0		0
20	b^5b^5	+	+			0	70		0	
21	b^9b^9	+	+			0	0		0	
22	b^9b^9	+	+			0	0		0	
22	b^9b^9			+	+		0	51		0
23	b^5b^9	+	+			0	61		0	
23	b^5b^9			+	+		39	12	0	21
11	b^4b^5				+			0		0
14	b^4b^5	+	+			52	11		12	
14	b^4b^5	Abs.b5[b]	Abs.b4			56	13		8	
14	b^4b^5	Abs.b4	Abs.b5			0	0		0	
15	b^4b^5	+	+			50	5		16	
15	b^4b^5	Abs.b4	Abs.b5			0	0		0	

[a] Single aliquots of cells from either Peyer's patch or peripheral blood were stained with both of each pair of reagents.

[b] Fluorescent anti-allotype reagents were passed through small Sepharose-IgG columns of the indicated allotype. Volumes of immunoadsorbents and reagent concentrations and volumes were standardized for all absorptions.

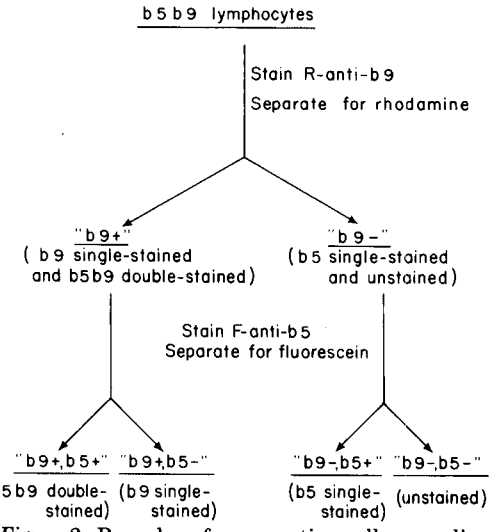

Figure 2. Procedure for separating cells according to membrane allotype, using the fluorescence-activated cell sorter. Peyer's patch or peripheral blood lymphocytes were first stained and separated for one allotype, resulting in fractions enriched (*left*) or depleted (*right*) for cells bearing that allotype. These two fractions were then stained and separated for the second allotype. The expected content of each fraction is given.

and the cell sorter; visual detection with the microscope is probably more sensitive to R fluorescence than to F fluorescence, while the reverse is true for the cell sorter.

The chief obstacle to isolating more highly purified fractions of cells bearing one allotype or the other on their membranes, however, was the high proportion of cells in the initial population which stained for both allotype markers. A series of experiments was undertaken in an attempt to obtain cell preparations depleted of double-stained cells and to explore their origin and significance.

Stripping of membrane Ig. The incubation of rabbit lymphocytes with 0.25% Pronase for 30 min at 37°C effectively eliminated their ability to be stained with fluorescent anti-allotype reagents (Table IV). After overnight culture the membrane allotype determinants reappeared on Pronase-treated cells so that the proportion of total cells staining for any allotype marker was comparable to that in cultures of MEM-treated (control) cells, and at least as great as in the original untreated cells. However, the percentage of cells which double-stained for two allotypes did not return to original levels, but instead remained at 2% of total lymphocytes for b4b5 cells and 6 to 7% for b5b9 cells. It should be noted also that, after culture, the proportion of cells treated with MEM alone which double-stained was also lower than before culture, the difference being greater in experiments with cells from b^5b^9 rabbits than in those in which cells from b^4b^5 rabbits were used.

Because these observations were made at arbitrary times after treatment with Pronase or with MEM alone, it could be argued that the cells had not been cultured long enough for a minority determinant to be present in sufficient quantities to be detected. Therefore, the time course of reappearance of membrane allotype markers was determined. Figure 4 gives the total proportion of MEM-treated and Pronase-treated cells which stained for allotype, and the proportion which double-stained for both b4 and b5 after varying periods of culture, in three experiments. Although membrane allotype markers began to re-appear as early as 4 hr after Pronase stripping and reached normal levels at least by 12 hr of culture, the proportion of Pronase-treated cells which double-stained remained under 5% after 2 and 3 days in culture. In addition, the proportion of double-stained cells in control cultures seemed to approach the level observed in cultures of Pronase-treated cells.

Separation of Pronase-stripped lymphocytes. Since treatment of lymphocytes with Pronase, followed by culture, significantly reduced the proportion of cells double-staining for two allotypes, this procedure was used to prepare cells for separation with the fluorescence-activated cell sorter. Peyer's patch cells from a b^5b^9 rabbit were stripped with Pronase and put into culture (Fig. 5*a*). After 13 hr in culture there were no cells double-stained for both b5 and b9, a substantial decrease from the 15% present in MEM-treated cells before culture. The Pronase-treated cells after culture were then stained and separated on the cell sorter, first for b9 and then for b5 (Fig. 5*b*). The resulting four fractions showed excellent purification of the two classes of single-stained cells. As expected, there were no cells in the doubly-enriched ("b9+, b5+") fraction, which would have contained the double-stained cells.

Binding of serum Ig to peripheral blood lymphocytes. The lack of reappearance, in Pronase-treated cells after culture, of the same high

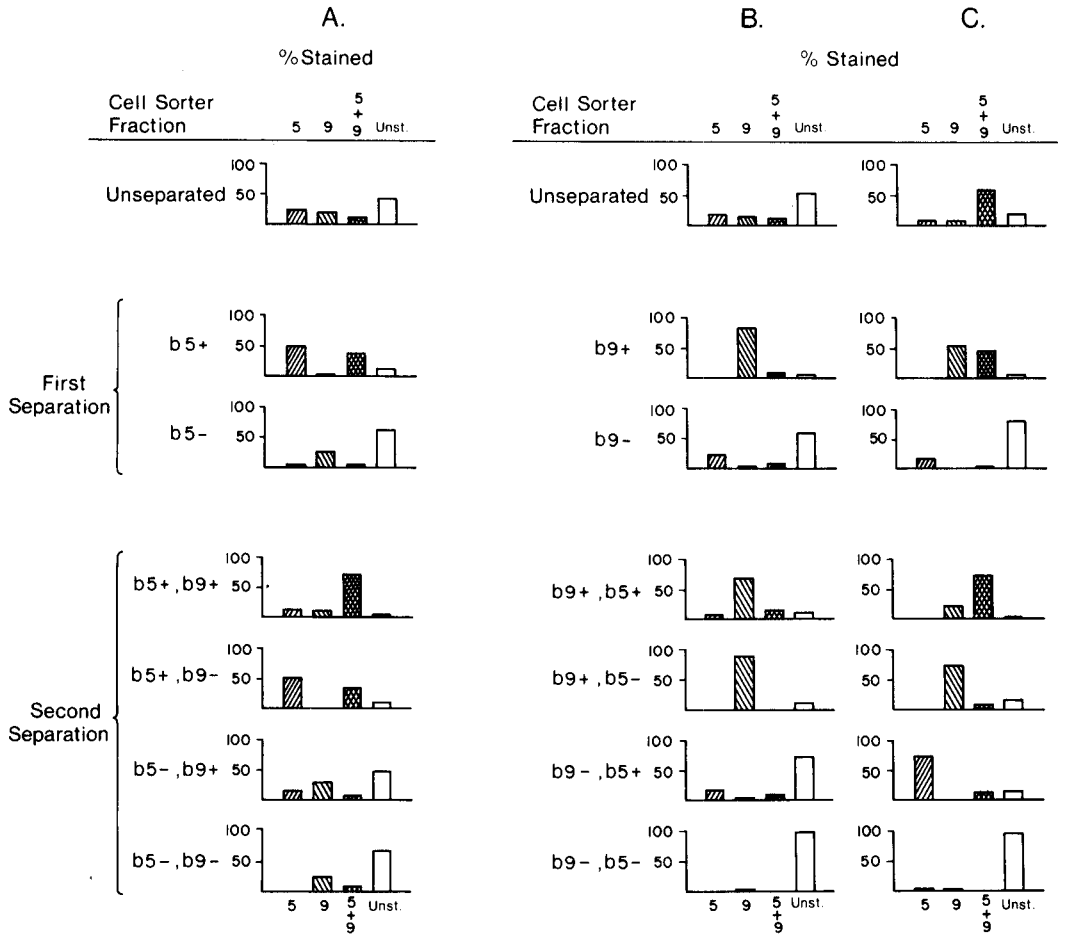

Figure 3. Separation of lymphocytes from b^5b^9 rabbits according to membrane allotype in three experiments. *A*, Peyer's patch cells (rabbit 4) were stained with F-anti-b5, separated under conditions optimal for detecting fluorescein, then stained with R-anti-b9 and separated under conditions for detecting rhodamine; *B*, Peyer's patch cells (rabbit 5); and *C*, peripheral blood cells (rabbit 6) were stained with R-anti-b9, separated under conditions for rhodamine, then stained with F-anti-b5 and separated for fluorescein. A portion of the unseparated cells in each experiment were stained with both reagents to obtain a staining analysis of the original lymphocyte population.

levels of cells bearing two allotype markers that was present in cells taken directly from the donor suggested that these cells probably had not synthesized both types of molecules but perhaps had picked up exogenous Ig *in vivo*. For exploring this possibility, peripheral blood lymphocytes were incubated in sera and then stained with fluorescent anti-allotype reagents for the detection of bound Ig. The results of three representative experiments are given in Table V. Staining lymphocytes from b^9b^9 donors after incubation in 50% b4b5 serum in MEM demonstrated clearly that many lymphocytes can bind serum Ig, whether exposed to the serum at 4°C for 30 min (experiment 1), or at 37°C for 30 min, or in culture for 21 hr (experiment 2). The appearance of these stained cells under the fluorescence microscope was not detectably different from that of routinely stained b4b5 cells, showing again a wide range of size and number of fluorescent patches and, on double-stained cells, of proportions of the two allotypes. The main deviation from normal staining patterns was the absence of b5 single-stained cells. Staining separate aliquots of untreated cells with R-anti-b9 revealed that rab-

TABLE IV

Stripping and reappearance of membrane Ig on peripheral blood lymphocytes from b^4b^5 and b^5b^9 rabbits

Cells[a]	% Stained							
	b4	b5	b4b5	Sum	b5	b9	b5b9	Sum
Untreated	44.2 ± 7.4[b]	5.5 ± 2.7	14.0 ± 2.4	63.8 ± 7.8	ND[c]	ND	ND	ND
MEM treated								
Before culture	40.5 ± 9.6	4.5 ± 1.1	11.0 ± 2.5	58.5 ± 5.7	a) 7[d]	1	41	49
					b) 19	12	45	76
After culture	50.8 ± 3.0	13.0 ± 4.8	8.0 ± 1.5	71.8 ± 6.3	a) 5	7	28	40
					b) 32	24	9	65
Pronase treated								
Before culture	0	0	0	0	a) 2	3	0	5
					b) 0	1	0	1
After culture	63.0 ± 4.3	15.0 ± 4.1	2.2 ± 0.5	80.2 ± 6.0	a) 14	21	6	41
					b) 40	34	7	81

[a] Peripheral blood lymphocytes were either stained for allotype without treatment ("untreated") or were incubated for 30 min at 37°C in MEM alone ("MEM-treated") or in MEM + 0.25% Pronase ("Pronase-treated") before being stained or put into culture. After culture (19 to 22 hr for b4b5 cells, 13 or 15 hr for b5b9 cells) samples again were stained.

[b] Means ±1 standard error for four experiments.

[c] ND, not determined.

[d] Data from two experiments.

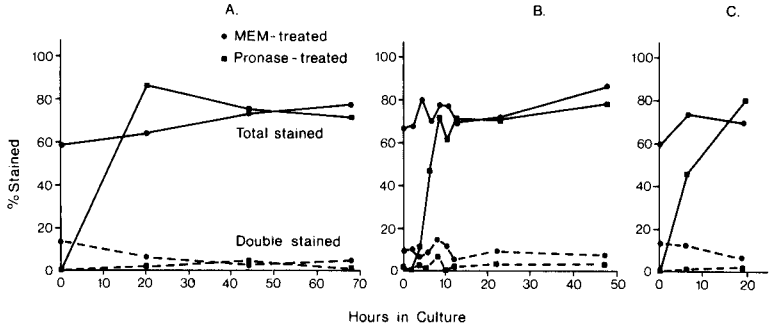

Figure 4. Membrane staining of pronase-treated (■) and MEM-treated (●) b4b5 peripheral blood lymphocytes after varying periods of culture, in three experiments. ——, percentage of lymphocytes stained for allotype (sum of percentage of single and double-stained); - - -, percentage of double-stained for both b4 and b5.

bits 22 and 21 had 51% and 47% of their cells, respectively, staining for their own allotype, so that at least as many cells were capable of binding exogenous Ig as had their own membrane Ig. An additional sample of cells from rabbit 21 after 21 hr in culture with 10% b4b5 serum was stained with F-anti-b5 and R-anti-b9, yielding 18% double-stained for both b5 and b9, 26% b9 single-stained, and no b5 single-stained cells. This is further evidence that cells which bind serum Ig also have membrane Ig of their own allotype.

In experiment 3, b4b5 cells were treated at 37°C for 30 min with MEM alone or with Pronase. Samples of untreated, MEM-treated, and Pronase-treated cells were then stained for b4 and b5 either directly or after 30 min incubation in 50% b4b5 serum; the rest were put into culture. After 22 hr in culture, samples of MEM-treated and Pronase-treated cells were again stained for allotype directly or after incubation in serum. Table V shows that the proportion of double-stained cells in untreated or MEM-treated populations could be increased by the exposure to serum, indicating that the cells had bound serum Ig. Treatment with

Pronase removed most of the sites of binding, since immediately after stripping only 6 to 7% of the cells picked up Ig. After 22 hr in culture the stripped cells were again capable of binding serum Ig; serum incubation restored the proportion of double-stained cells to the level in the untreated population. Autologous serum, obtained 4 days before the experiment, reacted with the cells of rabbit 10 as well as did serum from rabbit 24, indicating that the binding of serum Ig was not due to the presence of alloantibodies in rabbit sera.

Because of evidence in other systems that pre-formed antigen-antibody complexes can be bound by lymphocytes, a preliminary attempt was made to determine if the binding of complexes was responsible for the results described here. To the serum of rabbit 10, which was obtained 6 weeks after the rabbit was immunized with ovalbumin (OA) in CFA and which showed a strong reaction with OA in Ouchterlony plates, was added sub-precipitating amounts of OA. This preparation, which should have been enriched in antigen-antibody complexes, did not bind to the cells of rabbits 21 and 10 in a consistently different manner than did the serum without OA. Even cells that had been Pronase-stripped and cultured, whose membranes should not have been saturated with bound exogenous Ig or complexes, did not show a higher proportion staining for allotype after incubation with serum + OA than with the serum alone. While these results do not rule out the pick up of complexes by rabbit lymphocytes, it did not appear to be the major cause of the binding of serum Ig to cells in these experiments.

DISCUSSION

There is considerable disagreement in the literature as to whether or not individual lymphocytes are restricted to the expression of a single polymorphic form of Ig on their membranes. While some studies with a variety of species have shown that the vast majority of cells bear molecules of only a single class, type,

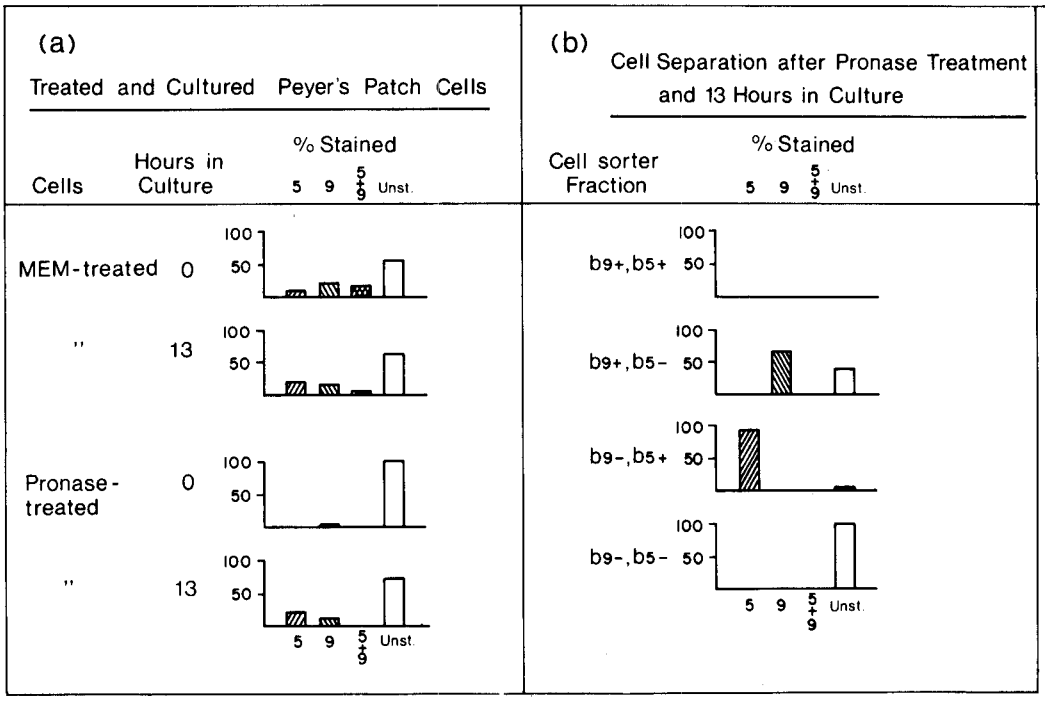

Figure 5. Cell separation of pronase-stripped and cultured b5b9 Peyer's patch lymphocytes. *a*, Cells were treated with pronase or with MEM alone and then cultured for 13 hr; *b*, the cultures of pronase-treated cells were harvested, washed, stained and separated first with R-anti-b9 and then with F-anti-b5. The distribution of stained cells in the b9+ and b9− fractions resulting from the first separation was not determined.

TABLE V

Binding of serum Ig to peripheral blood lymphocytes

Experiment No.	Rabbit No. (and Allotype)	Cells[a]	Hours in Culture	b4b5 Serum[b] (from Rabbit No.)	% Stained			
					b4	b5	b4b5	Sum
1	22 (b^9b^9)	Untreated	0		0	0	0	0
			0	24	18	0	58	76
2	21 (b^9b^9)	Untreated	0		0	0	0	0
			0	10	26	0	20	46
			0	10 + OA	17	3	24	44
		Untreated	21	10[c]	30	0	15	45
			21	10 + OA[c]	38	0	14	52
3	10 (b^4b^5)	Untreated	0		36	10	10	56
			0	10	22	0	38	60
			0	24	36	3	19	58
		MEM treated	0		26	6	8	50
			0	10	23	3	49	75
			0	24	56	2	21	79
		MEM treated	22		51	21	10	82
			22	10	31	0	67	98
			22	10 + OA	53	2	35	90
			22	24	52	4	32	88
		Pronase treated	0		0	0	0	0
			0	10	4	2	0	6
			0	24	4	1	2	7
		Pronase treated	22		63	20	2	85
			22	10	69	9	17	95
			22	10 + OA	51	17	19	87
			22	24	58	11	19	88

[a] Peripheral blood lymphocytes were put into culture without prior treatment ("untreated") or after 30 min at 37°C in MEM alone ("MEM-treated") or in MEM + 0.25% pronase ("pronase-treated"). Both before and after culture, samples of untreated, MEM-treated, and pronase-treated cells were stained for b4 and b5 either directly or after exposure to b4b5 serum.

[b] With the exception of cultured cells in experiment 2,[c] cells were incubated before or after culture in 50% b4b5 serum from rabbit 24 (unimmunized) or rabbit 10 (immunized 6 weeks previously with OA in CFA) to which 4 μg/ml OA was added as indicated. Incubations with serum were for 30 min at 4°C (experiment 1) or at 37°C (experiments 2 and 3). Cells were then washed three times at 4°C before being stained with F-anti-b4 and R-anti-b5.

[c] Cells were cultured for 21 hr in the presence of 10% b4b5 serum from rabbit 10, with or without OA. After culture these cells were then stained for allotype without further exposure to b4b5 serum.

or allotype (2, 27–30), others have shown the presence of determinants of several molecular forms of Ig on individual cells, by using similar techniques (6, 31–33). In agreement with this lack of restriction have been the results of Sell, Gell and co-workers, using anti-Ig reagents to stimulate rabbit lymphocytes to undergo blast transformation (34–36). Thus, in contrast to individual plasma cells, whose cytoplasm contains only a single kind of molecule (37–39), the lymphocyte precursors of antibody forming cells might not yet be so restricted.

Among the studies of *b* locus allotype markers on rabbit lymphocytes, our observations of high

proportions of double allotype-staining cells in peripheral blood (7 to 63% of total lymphocytes) and Peyer's patch (5 to 15%) were very similar to those of Wolf *et al.* (32). Using the mixed antiglobulin (immunocytoadherence) technique they found that 3 to 69% of peripheral blood lymphocytes from b^4b^6 rabbits had both b4 and b6 on their membranes. However, other membrane staining studies have reported that no cells from heterozygotes stained for both allotypes (2). Davie *et al.* (28) using combined immunofluorescence and autoradiography found that a maximum of only 3.4% of peripheral blood lymphocytes from b^4b^5 rabbits carried two allotypes.

It is difficult to explain these discrepancies, although it is very probable that even the use of similar techniques can produce quite different results. We have found that fluorescence microscopes can differ greatly in their sensitivity to R or F fluorescence and that minor changes in microscope components can greatly affect results. If cells staining for two allotypes on their membranes usually have more of one marker than the other, as was generally the case in our experiments, then a technique not quite as sensitive might not detect them. This problem of sensitivity may have been responsible for the difference between b^5b^9 and b^4b^5 rabbits in the proportion of peripheral blood cells which double-stained for two allotypes: 60% of cells with membrane allotype from b^5b^9 rabbits (Table I) and 20% of Ig-bearing cells from b^4b^5 rabbits (Table II). Different microscopes were used to obtain the two sets of data. It is possible, also, that the reagents in the two staining systems differed in their sensitivities.

More detailed studies of the cells which membrane stained for allotype revealed that exogenous serum Ig can bind to a large proportion of rabbit lymphocytes *in vitro* and probably also *in vivo*. This uptake of molecules by cells is probably the origin of our double allotype membrane staining. The binding of serum Ig could be a function of either the cells or the serum; specific receptors on the cell surfaces could bind serum molecules, or there might be antibodies in rabbit sera which react with molecules on the membrane.

Lymphocytes have been reported to bind Ig by a number of different mechanisms. A receptor for complement (C) has been described in the mouse which binds particulate antigens, such as flagella or sheep erythrocytes, coated with antibody and complement (40, 41). The receptor seems to be found only on bone marrow-derived (B) lymphocytes but apparently is separate from the receptor for antigen (42, 43). Basten *et al.* (44, 45) found that Ig can be picked up by mouse lymphocytes in the absence of C by means of a receptor for Fc; the bond is very weak, however, and the Ig can be removed with a few washes unless stabilized by the presence of antigen. Similar C-independent receptors for Fc have been described by others, with the difference that the cells must be exposed directly to either antigen-antibody complexes (46) or to aggregated Ig (47) for binding to be detected; once bound, however, the Ig is not easily dissociated.

Alternatively, antibody molecules in the rabbit sera may recognize antigenic determinants on lymphocyte membranes. Cells obtained from lymphoid tissues would retain their coat of antibodies until replaced by the normal turnover of membrane components and hence might stain for multiple Ig determinants. One possibility is that these are anti-Ig antibodies as have been described for a number of species (48, 49), which might react with membrane Ig. Bokisch and co-workers (50) have reported the presence of large quantities of both 7S and 19S anti-Ig antibodies in the sera of hyperimmune rabbits. Mandy has identified three homoreactive antibodies, present in all rabbit sera, which react with normally hidden determinants in the hinge region of rabbit Ig (51–53), sites which conceivably could be exposed in membrane Ig.

Although it is not yet possible to explain our findings of serum Ig binding to rabbit lymphocytes in terms of any of these mechanisms, some observations are pertinent. Preliminary results indicate that complexes do not bind preferentially to rabbit lymphocytes. Serum containing complexes of OA and anti-OA did not seem to increase binding of Ig compared to the serum alone. The incubation of Peyer's patch cells with complexes of goat IgG and R-labeled rabbit anti-goat IgG globulin did not stain the lymphocytes (S. Craig and P. Jones, unpublished observations), even though Peyer's patch cells are capable of binding serum Ig. If binding is due to the presence of a receptor for C on rabbit lymphocytes, one might have expected an increase in binding with the serum containing OA-anti-OA complexes, which did not oc-

cur, but this possibility will have to be more thoroughly examined.

The proportion of peripheral blood lymphocytes in the rabbit which bind Ig (Table V) is high compared to mouse (44) and human (47) lymphoid tissues. In those species Ig binding is a property of B cells; this seems to be true also in the rabbit, in which a large proportion of peripheral blood lymphocytes are B cells. The staining data in Table V indicate that the percentage of b4b5 peripheral blood lymphocytes which stain for allotype is not increased by prior incubation in b4b5 serum, indicating that cells capable of binding serum Ig have their own membrane Ig. In experiments not reported here in which cells from rabbits of one allotype were incubated in serum of a different allotype, double staining for both markers substantiated this conclusion. Grey *et al.* (54) have found that some θ-positive lymphomas have receptors for IgG, but most studies to date indicate that thymus derived cells from normal animals do not bind large amounts of Ig.

The sites for binding of Ig are, for the most part, susceptible to Pronase digestion but reappear with time in culture. Only 6 to 7% of cells pick up noticeable amounts of Ig from serum immediately after stripping, but this fraction of cells increases with time, as reflected by the reconstitution of normal levels of double-staining cells with the exposure to serum (Table V). If the proportion of cells staining for both allotypes is taken as a rough index of numbers of cells with bound Ig on their membranes, then this binding is very stable *in vitro*. Cells have been kept in dilute suspension at 4°C for up to 8 hr without losing their ability to become double-stained. Also, after 1 day in culture at 37°C, the proportion of cells bearing two allotypes is still significantly higher for control (MEM-treated) cells than for Pronase-treated cells (Table IV).

With this stability in mind, it is interesting to note that prior Pronase stripping is not required for cells to be able to bind serum Ig. Even when incubated in 50% b4b5 serum at 4°C for 30 min, 76% of b9 lymphocytes from rabbit 22 (Table V) picked up b4 or b5 Ig. Similarly, the proportion of b4b5 cells which double-stained could be greatly increased by incubation in b4b5 serum without prior treatment with Pronase. These observations suggest that the sites of binding of serum Ig are not saturated in freshly obtained cells, even though the cells were bathed in serum *in situ*. This phenomenon is consistent with the binding of serum Ig being due to the reaction of anti-Ig antibodies in the serum with Ig on lymphocyte surfaces; further studies of this possibility are underway.

Several controls for binding of serum Ig to rabbit lymphocytes have been previously published. Wolf *et al.* (32) found that incubation of cells from b^4b^4 and b^6b^6 rabbits in sera from b^6b^6 and b^4b^4 rabbits, respectively, produced some mixed rosettes; 10% of cells with membrane Ig seemed to have both allotypes. Those authors, and Davie *et al.* (28) have also examined lymphocytes from baby rabbits at ages when the Ig in their sera was predominantly of maternal origin and allotype. In nearly all cases the allotype determinants on a neonate's cells corresponded to his own genotype, a finding which was interpreted by both groups as evidence that serum Ig does not bind to rabbit lymphocytes. However, as part of the same study Wolf *et al.* showed that even baby rabbits heterozygous at the *b* locus do not have cells bearing both allotypes, but that these cells increase in number with age until they reach up to 69% of total lymphocytes in the peripheral blood.

Thus cells from baby rabbits are not an appropriate control for the binding of serum Ig. These animals might not yet have cells with appropriate receptors, or perhaps their sera are deficient quantitatively or qualitatively in the molecules which bind to cells. Preliminary experiments in our laboratory with heterozygous baby rabbits support the second explanation. We confirmed that very few peripheral blood lymphocytes double-stain for two allotypes, but found that *in vitro* they resemble adult cells in their ability to bind serum Ig from the mother or from unrelated rabbits. In addition, incubation of another animal's cells in sera from baby rabbits did not result in the pick up of serum Ig.

As mentioned above, lymphocytes with more than one kind of Ig molecule on their membranes have been found in a number of species. Because of results presented here and other demonstrations of the binding of Ig to lymphocytes, it is important that studies of membrane Ig in their role as receptors for antigen include some evidence that the molecules being investigated were synthesized by the cells that bear them. It is not known whether or not exogenous

Ig on a lymphocyte's membrane, after reaction with anti-globulin antibodies, could trigger the same effects on the cell that are initiated by the cell's own membrane Ig (e.g., ref. 13–15, 34–36). Thus, in exploring the functional significance of membrane Ig, it is essential to know that the membrane molecules under consideration were synthesized by the cells that carry them. Culturing lymphocytes after removing cell surface Ig with Pronase, trypsin (55), antibody-induced modulation (1), or by allowing normal metabolic release in culture (56, 57), should allow the exclusive expression of the cells' own membrane Ig, which could then be identified by staining or by isolation and characterization.

However, it is possible that lymphocytes are capable of synthesizing more than one polymorphic form of Ig, but not at the same time. To examine this question, and to investigate the possible precursor-product relationship between the membrane Ig of lymphocytes and the secreted products of their plasma cell descendants, one must still prepare populations of lymphocytes purified according to membrane Ig. The fluorescence-activated cell sorter can now isolate fractions of cells bearing a given marker in sufficient yields for functional tests (19). We have succeeded in separating lymphocytes from rabbits heterozygous at the *b* locus according to their membrane allotype with a double pass after staining the cells with two contrastingly-labeled fluorescent anti-allotype reagents, and can now test them functionally. Preliminary cell transfer experiments using these purified populations have indicated that cell sorter fractions enriched for cells bearing a single allotype give rise predominantly to plasma cells synthesizing Ig of that allotype.

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