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Membrane-Associated Immunoglobulin of Rabbit Peripheral Blood Lymphocytes: Allelic Exclusion at the *b* Locus

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ABSTRACT **Light chain allotypic determinants were demonstrated on the surface of peripheral blood lymphocytes of rabbits. By means of a combined immunofluorescent and autoradiographic technique, a great majority of the individual lymphocytes of rabbits heterozygous at the *b* locus were shown to have detectable quantities of only one allotype. A small proportion of lymphocytes appeared to possess both allelic forms of L chains.**

Cells specifically participating in immune responses display a high degree of specialization. This has been shown most extensively for antibody-secreting cells where it appears that individual cells produce a homogeneous population of antibody molecules. Thus, such cells produce antibody of a single specificity (1) and of but one immunoglobulin class (2). Further, in instances in which allelic allotypic variants of the same immunoglobulin exist, all molecules produced by a given antibody-secreting cell possess the same allotypic marker (allelic exclusion (3)).

Thus far it has not been determined at what stage of differentiation of immunocompetent cells this high degree of specialization is acquired, principally because immunocompetent cells that are not secreting large amounts of immunoglobulin have been relatively difficult to study. It has recently been established through the use of mixed agglutination (4, 5), inhibition of rosette formation (6), immunofluorescence (7), and radioautography (7, 8) that many lymphocytes possess membrane-associated immunoglobulin molecules, which may act as receptors for antigen. These cells are especially important in immune responses as they presumably are those that are capable of being stimulated, rather than those that have already been stimulated. It is, therefore, especially important to determine whether lymphocytes that bear membrane-associated immunoglobulins exhibit allelic exclusion.

Until very recently, attempts to investigate the nature of the membrane-associated immunoglobulin have utilized the capacity of various anti-immunoglobulin antisera to stimulate synthesis of DNA and to induce blast transformation in cultures of rabbit peripheral blood lymphocytes. Sell and Gell utilized this technique with antiallotype sera (9) and concluded that a significant portion of peripheral blood lymphocytes in heterozygous rabbits may possess both allelic forms of immunoglobulin (10).

Abbreviation: PBS, phosphate-buffered saline.

The introduction of immunofluorescent and antiradiographic techniques for the study of membrane-associated substances now allows a more direct approach to this question. We report in this communication that individual small lymphocytes from the peripheral blood of rabbits heterozygous at the *b* locus (b4b5) generally possess detectable quantities of only one allelic form of L chains. A small minority (3.4% or less) of cells in adult rabbits appear to have both allelic forms of L chains on their surface.

MATERIALS AND METHODS

Experimental animals

All rabbits were progeny of parents of known genotype bred in closed colonies at the National Institutes of Health.

Isolation of lymphocytes

Blood was collected in 3.8% sodium citrate from the marginal ear vein. Lymphocytes were isolated by the methods of Boyum (11). Briefly, the citrated blood was layered over a solution of 12.5% diatrizoate (Hypaque-M, 90%, Winthrop Laboratories, New York, N.Y.) and 1.25% methyl cellulose (15 centipoise, Fisher Scientific Co., Fairlawn, N.J.) in phosphate-buffered saline, pH 7.2 (PBS). Erythrocytes aggregated and sedimented into the bottom layer. The unsedimented portion was then layered over a solution of 9.9% diatrizoate and 6.3% Ficoll (Pharmacia, Uppsala, Sweden) in PBS and centrifuged at 400 $\times$ *g* for 40 min. Lymphocytes, which collected near the interface, were recovered and washed three times in tissue culture medium (Minimum Essential Medium, Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% fetal bovine serum and heparin (50 units/ml). Approximately 1 $\times$ 10^6 nucleated cells were isolated from each ml of peripheral blood. Of these, 90% or more were lymphocytes; a variable number of erythrocytes were also present in the final preparation.

Preparation and labeling of antiallotype antibodies

The anti-b4 (2C74-5) and anti-b5 (88K-3) antisera were obtained by immunization of rabbits of $a^1a^1b^6b^6$ genotype with purified immunoglobulin-G prepared from pooled sera of $a^1a^1b^4b^4$ and $a^1a^1b^5b^5$ rabbits, respectively (12).

Anti-b4 allotype antibodies were specifically purified by first passing the antiserum over an agarose conjugate (13) of b5-IgG to remove any cross-reacting antibodies. The effluent was then allowed to absorb to a b4-IgG–agarose column. The anti-b4 antibodies were then eluted with a glycine·HCl

buffer (0.05 M, pH 2.8). Anti-b5 antibodies were purified in a similar manner, except that the specificity of the agarose columns was reversed.

The purified antibodies were labeled with ^{125}I by the chloramine T method (14). The [^{125}I]anti-b4 reagent had a specific activity of 43.8 μCi/μg and was diluted to 6.7 μg/ml. The [^{125}I]anti-b5 reagent had a specific activity of 35.7 μCi/μg and a concentration of 6.7 μg/ml.

Antibodies were conjugated with fluorescein isothiocyanate (Nutritional Biochemicals Co., Cleveland, Ohio) by the dialysis method of Clark and Shepard (15). The conjugates were not absorbed prior to use. Fl-anti-b4 and Fl-anti-b5 were diluted to 3.8 and 3.3 mg/ml, respectively, in PBS that contained sodium azide (1 mg/ml).

Staining of lymphocytes by labeled antiallotype antibodies

2–10 $\times$ 10^6 lymphocytes were suspended in 0.2 ml of tissue culture medium containing heparin (50 units/ml) and sodium azide (1 mg/ml). The cells were cooled to 4°C, and 33–67 ng (5–10 μl) of [^{125}I]antiallotype antibody was added. After 30 min of incubation, unbound antibody was removed by washing the cells 4 times through gradients that contained fetal bovine serum (16). If only radioactive label was employed, the cells were smeared on serum-dipped slides, air dried, and fixed for 30 min in methanol–acetic acid–water 89:1:10. In the experiments where fluorescent label as well as radioactive label was used, the cells were first resuspended in 0.2 ml of fluorescein-labeled, purified antiallotype antibody and incubated at room temperature for 30 min. The cells were washed two more times through gradients concontaining fetal bovine serum, smeared on slides, and fixed as described above. The slides were then dipped in Nuclear Track Emulsion (NTB-2, Eastman Kodak Co., Rochester, N.Y.) that had been diluted 1:2 in water and heated to 50°C. Slides were stored in a stainless steel container *in vacuo* with a desiccant for appropriate exposure times (2–10 days). The autoradiographs were developed using standard methods.

It should be emphasized that the cells were intact morphologically during incubation with both ^{125}I- and fluorescein-labeled antibodies. Further, the presence of sodium azide served to inhibit pinocytosis; we assume, therefore, that only membrane-bound allotypic determinants were detected.

Immunofluorescent autoradiography

The simultaneous examination of lymphocytes for fluorescence and silver grains was done on unstained preparations using an oil immersion, dark-field condenser. A fluorescence microscope (Ortholux, E. Leitz, Inc., New York, N.Y.) equipped with a high-pressure mercury lamp (HBO 200 W) was employed. All microscopic examinations were made at a magnification of $\times$540 under oil immersion. With a standard optical system for fluorescence (UG-1 excitation filter; K 410 barrier filter), fluorescein was green, whereas silver grains were not visible. Fluorescence could be considerably enhanced by excitation with ultraviolet light of a slightly higher wavelength (BG-12 excitation filter; K 510 barrier filter). Under these conditions fluorescein was intensely green and silver grains appeared as faint blue dots. Light from a tungsten source caused the silver grains to appear as white dots and fluorescein was not detected. In the evaluation of experimental slides, either the BG-12 excitation system and the tungsten light source were used sequentially, or a field was initially evaluated under ultraviolet light and then under both ultraviolet and tungsten light. Identical results were obtained with both systems. For black and white photography, Tri-X Pan film (TX-135, Kodak) was used. Best photographic results were obtained by using shorter wavelength ultraviolet light alone or with tungsten light. Thus, photographs reveal either fluorescence alone or fluorescence and silver grains. After fluorescent and autoradiographic examination, the cells were stained with methyl green–pyronin. Individual fields were relocated by stage coordinates and photographed on Panatomic X film (FX-135, Kodak).

RESULTS

Allotypic determinants of the *b* locus were specifically identified on the surface of rabbit peripheral blood lymphocytes by the use of ^{125}I-labeled antiallotype antibodies. In the experiments presented in Table 1, the percentage of cells bearing these determinants varied from 38 to 64%. Rabbits homozygous at the *b* locus (b4b4 or b5b5) possessed a larger percentage of cells that bound the relevant single antiallotype antibody (anti-b4 or anti-b5) than did rabbits heterozygous at that locus (b4b5). This is in accord with results recently reported by Jones *et al.* (8). Further, the use of anti-b5 antisera with cells from a b4b4 rabbit or anti-b4 sera with cells from a b5b5 rabbit resulted in 1.9 and 0.9%, respectively, of the cells displaying grains. In general, these positive cells had relatively few grains.

In each instance the use of anti-b4 and anti-b5 together, with cells from heterozygous (b4b5) rabbits, resulted in a percent of labeled cells essentially equal to the sum of percent of cells labeled by the independent use of the two reagents. This result suggests, then, that allelic exclusion is exhibited by a large fraction of peripheral blood lymphocytes. Nevertheless, the inherent difficulties of summative experiments are such that a minor population of cells bearing both determinants might easily be present and escape detection. Indeed, the variability encountered when peripheral blood cells from individual rabbits were obtained and examined at different times (Table 1, Experiment 3) emphasizes these difficulties. However, these experiments strongly suggest that the cell-associated immunoglobulin is not merely passively coating these cells, as this should lead to virtually every cell possessing both allelic forms of immunoglobulin.

A more definitive way of evaluating the frequency of cells possessing both the b4 and b5 allotypic markers is the use of a double label. For this, we employed fluorescein and ^{125}I-labeled antiallotype antibodies. We initially established the specificity of the antibodies (Table 2). Thus, lymphocytes from a b5b5 (Experiment 6) and from a b4b4 (Experiment 7) animal were not stained by the antiallotype reagent directed at the *b*-locus-allotypic form of light chains which the rabbits did not possess. Furthermore, experiments using both ^{125}I- and fluorescein-labeled reagents of the same specificity (Experiment 8) demonstrated that the fluorescein-labeled reagents were only slightly more sensitive in detecting immunoglobulin-bearing cells than were the radioiodinated reagents.

The use of a radioiodinated and a fluoresceinated reagent of differing specificity in heterozygous rabbits (Experiments 8–10) clearly reveals that the bulk of cells bind only one of the two antiallotype antibodies (Fig. 1). Methyl green–pyronin staining showed that virtually all of these cells were small lymphocytes. Experiments on the lymphocytes of three

TABLE 1. *Binding of [^{125}I]antiallotype antibodies by rabbit lymphocytes*

Expt.	(Rabbit)*	Allotype	Reagent†	Total cells counted	% positive‡	c / a + b§	b4/b5 Cells¶	b4/b5 Serum‖
1	(264-4)	b4b4	(*a*)[^{125}I]anti-b4	663	64.3			
			(*b*)[^{125}I]anti-b5	705	1.9			
			(*c*) Both	633	60.1			
2	(262-3)	b5b5	(*a*)[^{125}I]anti-b4	677	0.9			
			(*b*)[^{125}I]anti-b5	705	62.4			
			(*c*) Both	705	53.8			
3	(262-2)	b4b5	(*a*)[^{125}I]anti-b4	661 (624)**	28.7 (22.3)	0.97 (1.36)	1.68 (3.33)	1.94
			(*b*)[^{125}I]anti-b5	705 (584)	17.1 (6.7)			
			(*c*) Both	691 (532)	44.6 (39.4)			
4	(262-4)	b4b5	(*a*)[^{125}I]anti-b4	588	32.4	1.01	2.89	2.23
			(*b*)[^{125}I]anti-b5	560	11.2			
			(*c*) Both	490	44.0			
5	(264-2)	b4b5	(*a*)[^{125}I]anti-b4	172	30.9	0.90	2.69	1.65
			(*b*)[^{125}I]anti-b5	218	11.5			
			(*c*) Both	128	38.2			

* Rabbits 264-2 and 264-4 were littermates, offspring of a b4b5 dam and b4b4 sire; rabbits 262-2, 262-3, and 262-4 were littermates, offspring of a b4b5 dam and b5b5 sire.

† Cells were treated with (*a*)[^{125}I]anti-b4, (*b*)[^{125}I]anti-b5, or (c) both [^{125}I]anti-b4 and [^{125}I]anti-b5.

‡ Cells having 2 grains or more are considered positive.

§ Percent of cells stained when both [^{125}I]anti-b4 and [^{125}I]anti-b5 are used (c) divided by sum of percentage of cells stained by each reagent separately (a + b).

¶ Ratio of cells bearing b4 allotypic determinants to cells bearing b5 allotypic determinants.

‖ Ratio of serum immunoglobulin molecules bearing b4 determinants to those bearing b5 determinants. Immunoglobulin concentrations were measured by a modified method of radial diffusion (17) on serum samples obtained from the rabbits donating the cells.

** Figures in parentheses found in lymphocytes of rabbit 262-2 in an experiment performed 2 months after the initial experiment.

heterozygous rabbits, two adults and one which was 3 weeks of age, revealed a maximum of 3.4% of the total cells which bound both antiallotype reagents. Furthermore, studies of the cells of rabbit 264-1 (Experiment 8), in which the anti-b4 and anti-b5 antibodies were used in both fluoresceinated and radioiodinated forms in alternate experiments, revealed a considerable degree of reproducibility of the percentage of cells bearing each immunoglobulin and of those bearing both. The relative proportion of cells stained by the specific reagents closely parallel the relative serum concentrations of immunoglobulin in normal adult heterozygous rabbits bearing b4 and b5 light chains.

Examples of cells bearing both allotypic markers are shown in Fig. 2. Methyl green–pyronin staining revealed that approximately half of these cells were small lymphocytes, whereas the remainder were monocytes or cellular debris. A few of the doubly labeled lymphocytes contained small numbers of grains and probably represented the minor

TABLE 2. *Binding of [^{125}I]- and fluorescent-antiallotype antibodies by rabbit lymphocytes*

Expt.	Rabbit, allotype	Antibody specificity* F1-	Antibody specificity* ^{125}I-	Total cells counted	% positive† F1 alone	% positive† Mixed	% positive† ^{125}I alone	% b4‡	% b5‡	b4/b5 Cells¶	b4/b5 Serum‖
6	Adult, b5b5 (262-3)	Anti-b4	Anti-b5	266	0	0	43.2	0	43.2	...	...
7	Adult, b4b4 (264-4)	Anti-b4	Anti-b5	142	47.2	0	0	47.2	0	...	...
8	Adult, b4b5 (264-1)	Anti-b4	Anti-b4	212	5.5	45.5	0.8	51.8	...	...	1.16
		Anti-b5	Anti-b5	136	4.6	26.9	1.0	...	32.5	...	1.16
		Anti-b4	Anti-b5	234	40.2	2.6	25.7	42.8	28.3	1.51	1.16
		Anti-b5	Anti-b4	349	26.5	3.4	36.2	39.6	29.9	1.32	1.16
9	Adult, b4b5 (262-2)	Anti-b4	Anti-b5	337	32.3	0.3	12.8	32.6	13.1	2.49	1.94
10	3-week-old, b4b5 (E128-1-1)	Anti-b5	Anti-b4	104	28.1	0	51.9	51.9	28.1	1.84	<.05

* Specificity of the fluorescein- and ^{125}I-labeled antibodies.

† Percent of individual cells labeled by fluorescence alone, by fluorescence and silver grains ("mixed"), or by silver grains alone; cells having 2 grains or more are considered positive.

‡ % b4 or % b5 is the total percentage of cells (doubly or singly labeled) reacting with antibody of that specificity.

¶ Ratio of cells bearing b4 allotypic determinants to cells bearing b5 allotypic determinants.

‖ Ratio of serum immunoglobulin molecules bearing b4 determinants to those bearing b5 determinants.

population that stained nonspecifically (Table 1, Experiments 1 and 2). However, the majority of doubly stained lymphocytes were intensely stained with both reagents.

Two further points deserve comment. First, the distribution of immunoglobulin on the surface of lymphocytes was rarely homogeneous (Fig. 1*A*). In most instances, a speckled pattern was noted that suggested that the immunoglobulin existed in small patches on the cell surface. In some instances, the speckled pattern was limited to only one region of the cell and the remainder of the cell was not stained. The small lymphocytes that bound both antiallotype reagents usually bound relatively large amounts of each antibody, and the two labels were either distributed on different regions of the cell (Fig. 2 *A* and *B*) or diffusely over the cell (Fig. 2 *C* and *D*).

Finally, the experiment performed on peripheral blood lymphocytes of a 3-week-old heterozygous rabbit (Table 2, Experiment 10) provided additional evidence that the immunoglobulin possessed by the cell did not derive from the serum. This animal was the offspring of the mating of a b5b5 doe and a b4b4 buck and consequently had a predominance of b5 (maternal) immunoglobulin due to placental transfer. Less than 5% of its circulating serum immunoglobulin was of the b4 type but 51.9% of its peripheral blood lymphocytes bore the b4 marker. On the other hand almost all of the serum immunoglobulin was of the b5 type but only 28.1% of the lymphocytes possessed the b5 marker on their surfaces.

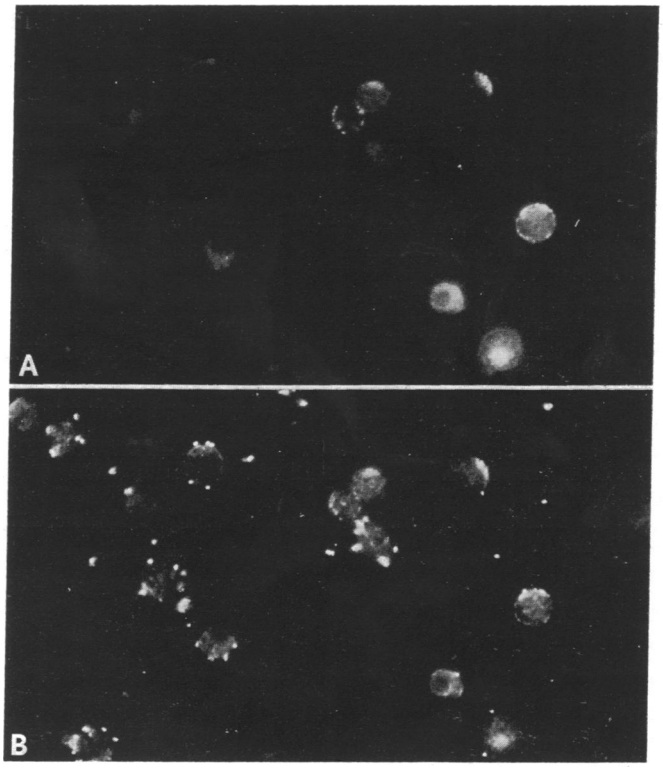

FIG. 1. Peripheral blood lymphocytes from a b4b5 rabbit (264-1) stained with ^{125}I-labeled anti-b4 and F1-labeled anti-b5. *A*, Cells examined with ultraviolet light alone. Fluorescent cells have b5 determinants. *B*, Same field examined with both ultraviolet and visible light. Cells with silver grains have b4 determinants. Fluorescent cells, of course, are still visible.

DISCUSSION

The data presented in this paper demonstrate that a great majority of immunoglobulin-bearing small lymphocytes in the peripheral blood of rabbits heterozygous at the *b* locus possess detectable quantities of only one of the allelic forms of L chains. Pernis *et al.* have recently obtained similar results for splenic and peripheral lymphocytes utilizing a double immunofluorescent label (18). These observations are somewhat different from those reported by Sell *et al.* in their studies of DNA synthesis and blast transformation of rabbit peripheral blood leucocytes exposed to antiallotype reagents (10). The conclusion reached by the latter authors was based upon the fact that mixtures of antiallotype sera caused the cells of heterozygous rabbits to display a greater response than the sum of the responses caused by the two reagents used separately. They reasoned that some cells existed that could not be stimulated by either reagent alone, but could be stimulated by the two reagents used together. It is possible that such cells would have too little immunoglobulin on their surface to be detected by either of the labeling reagents we employed and consequently would be counted as negative cells. A second possibility, which must also be considered, is that our studies are principally concerned with small lymphocytes, as they represent the bulk of the cells isolated by our methods, whereas Sell *et al.* utilized a more diverse collection of cells (10). It is possible that medium sized and large lymphocytes might display allelic exclusion less regularly than do small lymphocytes.

One must, of course, be most cautious in the use of the term allelic exclusion, which implies a total absence of the alternate form of immunoglobulin. All techniques have lower limits of sensitivity; it is possible that small amounts of the alternate immunoglobulin exist on many of the cells in which only a single allelic marker is detected.

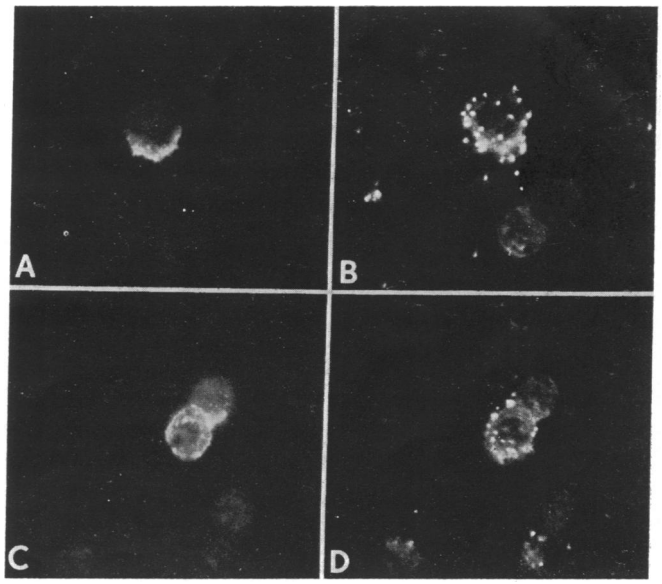

FIG. 2. Peripheral blood lymphocytes from b4b5 rabbit (264-1) stained with ^{125}I-labeled anti-b4 and F1-labeled anti-b5. *A* and *C*, Ultraviolet light alone; *B* and *D*, ultraviolet light and visible light. *A*, Lymphocyte with b5 determinants on one pole of cell. *B*, Same cell with b4 determinants distributed evenly over the surface. *C*, Cell with b5 determinant distributed evenly. *D*, Same cell with b4 determinants limited to half of the visible surface.

Finally, it is most intriguing that some cells do in fact bind both antiallotype reagents. Whether these represent cells that have not yet achieved the high degree of specialization characteristic of the bulk of cells is not certain. Nevertheless, these cells are of special interest and attempts to assess their significance are now in progress.

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