STUDIES ON RABBIT LYMPHOCYTES IN VITRO

VIII. The Relationship between Heterozygosity and Homozygosity of Lymphocyte Donor and Per Cent Blast Transformation Induced by Antiallotype Sera*

By STEWART SELL, M.D.

(From the Department of Pathology, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania 15213)

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Antisera to rabbit leukocytes (1) or to rabbit immunoglobulins (2–5) may stimulate cultures of rabbit peripheral lymphocytes. Such antisera stimulate lymphocytes in vitro to synthesize RNA, DNA, and protein, to transform into immature "blast" cells, and to undergo division (2). Allogeneic (rabbit) antisera to genetically controlled intraspecies antigenic determinants present on rabbit immunoglobulins (allotypes) (6–8) will also stimulate blast transformation of rabbit peripheral lymphocytes in vitro when the lymphocytes are obtained from an allotypically appropriate donor (9, 10). There are seven well-characterized immunoglobulin allotypes controlled by two chromosomal loci (7, 8, 10). Allotypes Aa1, Aa2, and Aa3 are controlled by the "a" locus and are located on the H chain of rabbit IgG; allotypes Ab4, Ab5, Ab6, and Ab9 are controlled by the "b" locus and are located on the L chain of rabbit IgG (7, 8, 10, 11).[1] Genetic observations indicate that the allotypes controlled by the a locus segregate independently of the allotypes controlled by the b locus (7, 8). A given rabbit may be homozygous or heterozygous for both loci or heterozygous for one locus and homozygous for the other locus. Specific antisera produced in rabbits to each of the seven above allotypes may stimulate blast transformation and DNA synthesis when added to in vitro cultures of lymphocytes from a donor of the appropriate allotypic specificity (12).[1]

Only one of the two genetically supplied allotypic determinants is present on a given immunoglobulin molecule (13) or in a given plasma cell of a heterozygous rabbit (14, 15). These findings indicate that there is inhibition of expression of one genetically controlled determinant with expression of the other determinant by antibody-producing cells (14, 15). If a similar control mechanism exists for the lymphocytes of a heterozygote, a given antiallotype serum

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(for example, anti-Ab4) might be expected to induce more lymphocytes to transform in cultures of lymphocytes obtained from homozygous donors (Ab4Ab4) than in cultures obtained from heterozygous donors (Ab4Ab5). However, a difference in the amount of blast transformation between lymphocyte cultures from homozygous or heterozygous donors induced by antiallotype sera could not be demonstrated previously (12). The present report describes experiments showing that the amount of blast transformation inducible in

TABLE I

Transformation of Homozygous Ab4 Ab4 Lymphocytes In Vitro by Anti-Ab4 Sera

Volumes of serum per culture*		Allotype of lymphocyte (donor)			
		a1 a1 b4 b4		a2 a2 b4 b4	
		Blast transformation	Thymidine-^{14}C uptake	Blast transformation	Thymidine-^{14}C uptake
	ml	%	*counts/10 min*	%	*counts/10 min*
Autologous	0.5	2	885	<1	1024
Autologous‡	0.5	>50	4338	>45	3720
Non-Ab4 normal	0.5	2	1009	—	—
Anti-Ab4	0.5	7	1419	5	1167
	0.25	15	1582	5	883
	0.12	29	1605	34	2789
	0.05	37	1923	15	1499
	0.025	62	2264	12	1467
	0.01	46	1548	—	—

* The total serum volume of all cultures was made up to 0.5 ml with normal non-Ab4 serum.

‡ 0.03 ml phytohemagglutinin M (Difco Laboratories, Detroit, Mich.—Control No. 481016) added.

cultures of lymphocytes from homozygous donors by the appropriate antiallotype serum is approximately twice as much as that inducible in cultures of lymphocytes obtained from heterozygous donors.

Materials and Methods

Lymphocyte Cultures. Blood was obtained from the marginal ear veins of healthy young adult rabbits of known immunoglobulin allotype and lymphocyte-rich suspensions obtained as described previously using 3% w/v pig skin gelatin (4, 9). The lymphocyte concentration of this suspension was determined by counting in a hemocytometer. The suspensions were washed once in Eagle's suspension medium containing $\frac{1}{10}$ volume Difco tryptose broth, 200 units/ml of penicillin and 100 units/ml of streptomycin. The lymphocytes were suspended in a volume of medium necessary to give a cell concentration of 5×10^6 lymphocytes per ml. 1 ml of this suspension was added to 2 ml of medium in bijou bottles (A. Gellenkamp and Co. Ltd., London, England) containing 0.5 ml of rabbit serum or 0.5 ml dilutions of rabbit anti-

allotype serum (see below). After incubation of the sealed cultures for 44 hr at 37°C each culture received 0.05 μc of uridine-^{14}C or thymidine-^{14}C. After the cultures had been incubated for an additional 4 hr, the lymphocytes were placed in suspension by gentle agitation of the cultures and 0.5 ml of the suspension removed. This aliquot was centrifuged at 1000 rpm for 10 min, smears made of the deposit of cells, and the smears stained with Jenner-Giemsa at pH 5.5 (9). The per cent of blast cells present was estimated from these smears by microscopic examination. The remaining volume of suspension in the culture was centrifuged at 1000 rpm for 10 min. The button of cells obtained was washed twice in 0.85% NaCl, allowed to dry, dissolved in 1.0 ml hyamine, and the radioactivity determined using a standard phosphore (9). The activity was recorded as disintegrations per 10 min.

Preparation of Antisera.—Non-Ab4 and non-Ab5 rabbits were immunized with the appro-

TABLE II

Transformation of Homozygous Ab5 Ab5 Lymphocytes In Vitro by Anti-Ab5 Sera

Volume of serum per culture*		Allotype of lymphocyte (donor)			
		a2 a2 b5 b5		a3 a3 b5 b5	
		Blast transformation	Thymidine-^{14}C uptake	Blast transformation	Thymidine-^{14}C uptake
	ml	%	*counts/10 min*	%	*counts/10 min*
Autologous	0.5	<1	454	<1	810
Anti-Ab5	0.5	2	788	1	1262
	0.25	3	593	3	1595
	0.12	72	1735	4	2245
	0.05	53	1076	29	2844
	0.025	77	2065	36	2453
	0.012	50	1384	31	5755

* The total serum volume of all cultures was made up to 0.5 ml with normal non-Ab5 serum.

priate γ-globulin allotype in the form of killed *Proteus vulgaris* organisms coated with anti-*Proteus* antibody, as described by Dubiski et al. (6). For example: an a1 a2 b5 b6 rabbit was injected with *P. vulgaris* coated with anti-*Proteus* antibody produced in an a1 a2 b4 b4 rabbit. Since the "a" locus determinants were matched and the immunized animal did not carry the Ab4 determinant, this animal produced a specific anti-Ab4 serum. Because of considerable variation in the blastogenic effect of different antiallotype sera only proven highly effective antiallotype sera were used in vitro. In addition, as the per cent blast transformation varies considerably with dose, all experiments were set up with a range of antiallotype serum dilutions.

RESULTS

To determine the relationship between the zygosity of the lymphocyte donor and the effect of antiallotype sera upon these lymphocytes in vitro, lymphocytes from homozygous b4b4, from homozygous b5b5, and from heterozygous b4b5 donors were cultured in vitro using anti-Ab4 and anti-Ab5 sera. 18 experiments using Ab4Ab4 donor cells cultured with anti-Ab4 sera; 12 experi-

ments using Ab5Ab5 donor cells cultured with anti-Ab5 sera; and 34 experiments using Ab4Ab5 donor cells cultured separately with anti-Ab4 and anti-Ab5 sera were performed.The results of representative experiments are shown in Tables I–III: Table I, Ab4Ab4 cells–anti-Ab4; Table II, Ab5Ab5 cells–anti-Ab5; and Table III, Ab4Ab5 cells–anti-Ab4 and anti-Ab5. The highest per

TABLE III

Transformation of Heterozygous Ab4 Ab5 Lymphocytes In Vitro with Anti-Ab4 and Anti-Ab5 Sera

Volume of serum per culture		Allotype of lymphocyte (donor)			
		a1 a2 b4 b5		a1 a3 b4 b5	
		Blast transformation	Thymidine-^{14}C uptake	Blast transformation	Thymidine-^{14}C uptake
	ml	%	*counts/10 min*	%	*counts/10 min*
Autologous	0.5	<1	403	<1	1210
Aa1 Aa1 Ab4 Ab4	0.5	<1	361	<1	1348
Aa3 Aa3 Ab5 Ab5	0.5	<1	448	<1	1467
Anti-Ab4	0.12*	7	632	3	2146
	0.05	22	701	7	2396
	0.025	11	682	15	2315
	0.012	—	—	25	2258
Anti-Ab5	0.12‡	6	511	1	1198
	0.05	11	634	12	1829
	0.025	8	673	6	1546
	0.012	14	832	5	2086

* Cultures containing anti-Ab4 serum were made up to 0.5 ml with non-Ab4 serum.

‡ Cultures containing anti-Ab5 serum were made up to 0.5 ml with non-Ab5 serum.

cent blast transformation induced in the 64 separate experiments is presented in graphic form in Fig. 1.

A given antiallotype serum stimulates approximately twice as many lymphocytes obtained from a homozygous donor to transform in vitro as lymphocytes from a heterozygote donor. The mean ± standard error of the mean (SEM) of the maximum per cent blast transformation for the various cultures are Ab4-Ab4–anti-Ab4, 39.78 ± 4.8; Ab4Ab5–anti-Ab4, 19.77 ± 1.6;Ab5 Ab5–anti-Ab5, 50.83 ± 7.0; and Ab4Ab5–anti-Ab5, 16.1 ± 1.6. The amount of DNA synthesis as determined by thymidine-^{14}C uptake ranges from two to five times the control (unstimulated cultures) in stimulated cultures made from homozygous donors, but is usually only twice the control in stimulated cultures from heterozygous donors. Essentially similar results have been obtained for the a

locus determinants, Aa1 and Aa2, but only a limited number of experiments directed toward these a locus determinants have been performed.

There is marked variability in the maximum per cent blast transformation from one experiment to another. This variability appears to be inherent in the nature of the experimental technique since there is, as yet, no apparent factor such as age, sex, condition of donor, etc., that correlates with a high or low response of the cultures. Recording the response of the cultures as the maximum

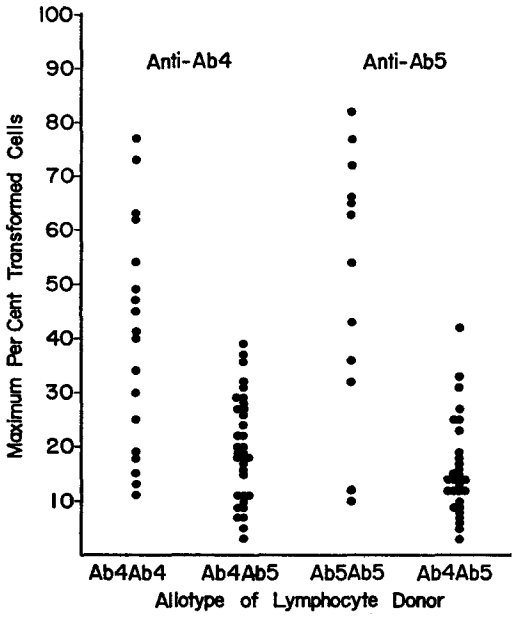

FIG. 1. The relationship between zygosity of lymphocyte donor and maximum per cent blast transformation induced with antiallotype sera. There is a marked variability of maximum per cent blast transformation from one experiment to another. Approximately twice as many lymphocytes obtained from homozygous donors transform as lymphocytes from heterozygous donors.

per cent or as maximum increase in thymidine-^{14}C uptake controls to some extent the variability of response due to concentration (dilution) of antiserum. Quantitation of the serum immunoglobulin allotype levels of the experimental animals by hemagglutination inhibition did not reveal a low concentration of a given allotype in the serum of those donors whose lymphocytes gave a low per cent transformation when stimulated in vitro. In addition, while repeat cultures of lymphocytes from a donor whose lymphocytes reacted well on first stimulation tended to give high percentages when restimulated, this was not a consistent result. Three of the homozygous donors were used twice while six of the heterozygous donors were reused.

DISCUSSION

The control of expression of immunoglobulin allotypy appears to be similar for plasma cells and for lymphocytes. Plasma cells have been demonstrated to be the primary cell responsible for the secretion of immunoglobulins. Fluorescent antibody studies have shown that a given plasma cell contains and probably produces immunoglobulins bearing only one of the two possible allotypic specificities controlled by a given locus in a heterozygote rabbit (14, 15). Thus, Pernis et al. (14) found that about 90% of the plasma cells in a b4b4 rabbit stained with fluorescent anti-Ab4, while in two b4b5 rabbits 61 and 53% of the plasma cells stained with fluorescent anti-Ab4 and 39 and 47% with fluorescent anti-Ab5. Very few, if any, plasma cells were found to contain both Ab4 and Ab5. Although it is structurally possible for an immunoglobulin molecule to contain two allotypic sites, a given immunoglobulin molecule of a heterozygous rabbit contains the same allotypic specificity at both sites (13). Therefore, allotypically mixed immunoglobulin molecules or plasma cells have not been demonstrated.

The present data illustrate that an anti-Ab4 serum can induce up to about 80% of the lymphocytes from an b4b4 rabbit to transform, but only a maximum of 40% of the lymphocytes from a b4b5 rabbit. Similarly anti-Ab5 sera can induce up to 80% transformation in Ab5Ab5 lymphocyte cultures and up to 40% in Ab4Ab5 cultures. From present and from previous data (9, 12) it is reasonable to speculate that most lymphocytes carry allotypic determinants and that the lymphocytes of a heterozygote express or carry only one of the two allotypic specificities supplied genetically. The failure to demonstrate this difference in the amount of blast transformation as related to the zygosity of the lymphocyte donor in previous studies (12) may have been due to an inadequate number of experiments in view of the marked variability of maximum per cent transformation from one experiment to another or to the use of antiallotype sera that would not produce maximal transformation. The expression of only one of two codominant alleles controlling antigenic specificities does not hold for all systems as both parental histocompatibly antigens are present in antibody-forming cells (16).

The ability of anti-immunoglobulin and antiallotype sera to induce transformation is difficult to explain unless the lymphocyte carries immunoglobulin and allotypic determinants, presumably on its surface. Since antisera to each of the subunits of IgG will induce high percentages of lymphocytes to transform in vitro it has been concluded that each lymphocyte carries determinants representative of the complete IgG molecule (4). The finding that xenogeneic antisera specific for different rabbit immunoglobulins (IgA, IgG, and IgM) induce transformation at high rates (5) deserves further comment in view of the possibility that plasma cells may produce immunoglobulin or antibody primarily for export while lymphocytes may produce immunoglobulin primarily

for individual use. The maximum per cent blast transformation induced by antisera of different specificities tested in this and in previous studies is given in Table IV. The high percentages induced with anti-IgG, anti-IgM, and anti-IgA sera were interpreted to indicate that all lymphocytes contained determinants of IgG and IgM; and approximately ⅓ of these lymphocytes contained determinants of IgA as well (5). This interpretation is inconsistant with observations that plasma cells contain only one immunoglobulin group at a time. However, occasional cells containing more than one immunoglobulin

TABLE IV

Maximum Blast Transformation with Antisera

Antisera to	Blasts
	%
Allotype b4*	77
Allotype b5*	82
Allotype b4‡	39
Allotype b5‡	42
L chain	91
γ-chain	88
μ-chain	81
α-chain	32
Fab	86
Fc	79

* Homozygous Ab4 Ab4 or Ab5 Ab5 lymphocytes.

‡ Heterozygous Ab4 Ab5 lymphocytes.

group have been identified in the germinal centers of lymphoid tissue (14, 15). The morphologic classification of these cells is uncertain. In addition, recent studies in vitro of lymphoid cell lines derived from patients with myelogenous leukemia and lymphocytic leukemia demonstrate that cultures of these lines often produce more than one immunoglobulin class (17–20).

The observation that plasma cells contain immunoglobulin of only one class while lymphocytes may contain immunoglobulins of more than one class may have at least two explanations: (*a*) The sensitivity of lymphocyte transformation by antisera is much greater than that of the fluorescent antibody technique and the inability to find more than one immunoglobulin group in a given plasma cell is simply a matter of technique. This is supported by the difficulty in identifying immunoglobulins in lymphocytes by the fluorescent antibody technique even though lymphocytes are transformed at high rates with anti-immunoglobulin sera (2). (*b*) Both plasma cells and lymphocytes may have the capacity to produce all types of immunoglobulin molecules, but not more than

one type at the same time. Immunoglobulins produced by plasma cells may be rapidly secreted so that at any given time only one immunoglobulin group may be identified in the cell. On the other hand, if the immunoglobulins produced by lymphocytes are not secreted or are secreted very slowly and remain in or attached to the lymphocyte, then different immunoglobulin groups produced at different times may be identified.

The ultrastructural features of plasma cells and of lymphocytes are consistent with a concept of immunoglobulin secretion by plasma cells and immunoglobulin retention by lymphocytes. Plasma cells contain considerable amounts of endoplasmic reticulum, a subcellular structure associated with the production of protein intended for secretion, while lymphocytes contain little, if any, endoplasmic reticulum (see review by Feldman, reference 21). The idea that lymphocytes might retain their immunoglobulins or release them much more slowly than plasma cells was adumbrated by Hummeler et al. who incriminated lymphocytes with little or no endoplasmic reticulum as antibody-releasing cells (22). Such cells were identified by the lysis in agar technique of Jerne and Nordin (23) and their ultrastructure characterized by electron microscopy (22). It is possible that lymphocytes with little or no endoplasmic reticulum might not be able to release their own immunoglobulins under normal conditions in vivo, and that their identification as antibody-secreting cells is an artifact of the in vitro plaque technique. On the other hand, Cooperband et al. (24), finding normal immunoglobulin synthesis and response to phytohemagglutinin by lymphocyte cultures obtained from patients with congenital agammaglobulinemia, speculated that the trace amounts of immunoglobulins found in the serum of such patients might have been produced by their lymphocytes.

The possibility that lymphocytes produce immunoglobulins or antibodies that remain associated for long periods of time with the cell that made them is an attractive explanation for the long-lived immunological specificity of lymphocytes (25, 26).

SUMMARY

Rabbit antisera to immunoglobulin allotype Ab4 will stimulate a maximum of 77% (mean ± SEM = 39.78 ± 4.8) blast transformation of Ab4Ab4 homozygous lymphocytes in vitro and a maximum of 39% (mean ± SEM = 19.77 ± 1.6) blast transformation of heterozygous Ab4Ab5 lymphocytes. Similarly anti-Ab5 sera will induce a maximum of 82% (mean ± SEM = 50.83 ± 7.0) blast transformation of Ab5Ab5 homozygous lymphocytes in vitro and a maximum of 42% (mean ± SEM = 16.1 ± 1.6) blast transformation of heterozygous Ab4Ab5 lymphocytes. Thus, the lymphocytes of an allotypically heterozygous rabbit appear to be selected to produce or express only one of the two genetically supplied allotypic determinants controlled by

the "b" locus. A similar conclusion has been made in regard to allotypic expression by immunoglobulin-producing plasma cells.

Previous data indicate that each lymphocyte carries or expresses more than one immunoglobulin group specificity (IgG, IgA, IgM) while each plasma cell carries only one. One possible interpretation of these data is that, while the production of immunoglobulin groups and allotypic specificities are under similar control in both the lymphocyte and the plasma cell, the lymphocyte produces immunoglobulin that remains in or attached to itself and the plasma cell produces immunoglobulins that are secreted rapidly.

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