STUDIES ON RABBIT LYMPHOCYTES IN VITRO

I. Stimulation of Blast Transformation with an Antiallotype Serum*

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The circulating lymphocyte has recently been reported to have many possible functions. Though once believed to be short-lived end cells (1), lymphocytes present in the peripheral blood are now known not only to recirculate for up to 5 days, but also to be capable of "homing" in lymph nodes and in the spleen (2). These cells have been reported to be capable of producing antibody (3), of transforming into plasma cells (4–6), of eliciting the graft *versus* host reaction (7, 8), of preventing the neonatal thymectomy wasting syndrome (9, 10), and in some situations of preventing the effects of lethal irradiation (11), and of repopulating the lymphoid tissues of irradiated animals (12).

In vitro studies demonstrate that human lymphocytes can be non-specifically stimulated to transform into "blast" cells with agents such as phytohaemagglutinin, staphylococcal filtrate, or streptolysin S and specifically stimulated with antilymphocyte antibody, or with specific antigen when this is added *in vitro* to cells from sensitized donors (see review by Robbins, 13). *In vitro* mixtures of cells obtained from 2 genetically different individuals are also capable of undergoing transformation (14–16).

Genetically determined differences in the antigenecity of 7S γ-globulin (IgG) have been identified in the rabbit (17). These differences can be recognized when a rabbit lacking a given γ-globulin antigen is immunized with the γ-globulin of another rabbit that contains the given antigenic determinants. These antigens have been termed allotypes. Six different allotypes have been identified, and breeding experiments indicate that these 6 allotypes are controlled by 2 chromosomal loci. The first locus, "a," controls allotypes As1, As2, and As3; the second locus, "b," controls allotypes As4, As5, and As6 (17).

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The present paper describes the induction of blast transformation in lymphocytes obtained from the peripheral blood of rabbits when cultured *in vitro* with antiallotype serum. This transformation requires specific antiallotype antibody, is not dependent on complement, and does not occur with mixtures of viable cells from allotypically different rabbits even if 1 donor is specifically immunized against an allotype present in the donor of other cells.

Materials and Methods

Animals.—Healthy adult rabbits were used throughout. The γ-globulin allotype of each rabbit was determined by specific reference antisera using double diffusion in agar (18).

Preparation of Antisera.—Each of 3 adult male rabbits of allotype As1,3,5,6, and 1 of allotype As1,4 were immunized with γ-globulin allotype As1,4, in the form of killed *Proteus vulgaris* organisms coated with antiproteus antibody produced in an As1,4 rabbit, as described by Dubiski, *et al.* (19). Sheep anti-rabbit γ-globulin, goat anti-rabbit γ-globulin, and guinea pig anti-rabbit γ-globulin sera were prepared according to standard techniques and the specificity of these antisera tested by gel diffusion.

Non-Specific Stimulants.—Bactophytohaemogglutinin M (PHA lot 0528-57, Difco Laboratories Inc., Detroit) and staphylococcal filtrate (SF) prepared as described by Ling *et al.* (20) were used.

Leucocyte Suspensions.—Blood was obtained from the marginal ear vein of rabbits after shaving and washing of the ear with 70 per cent ethanol. The whole blood was collected in 40 ml sterile, siliconized screw top bottles, and defibrinated immediately after collection with sterile wooden sticks. After defibrination, leucocyte-rich serum was obtained by adding one half volume of 3 per cent w/v pig skin gelatin (British Glues and Chemicals Ltd., London) and allowing the red cells to sediment for 1 to 2 hours at 37°C (21). The supernate leucocyte-rich serum was drawn off with a sterile Pasteur pipette and the white and red cell concentration determined by microscopic counting in a standard hemocytometer. The leucocyte-rich serum was then centrifuged for 10 minutes at 1200 RPM and the cell-free serum-gelatin supernate removed. The cells remaining were washed once in half the original volume of warm Eagle's medium (containing 1/10th volume of tryptose broth, 200 units/ml of penicillin and 100 units/ml of streptomycin) and then resuspended in the necessary volume of warm medium to give the desired cell concentration. Such leucocyte medium preparations usually had approximately 75 per cent erythrocyte contamination.

Leucocyte Cultures.—One ml of leucocyte suspension containing approximately 1.0×10^6 white cells per ml was added to 2.0 ml of warm medium in bijou bottles. Each bottle then received 0.5 ml of the appropriate normal rabbit serum supernate or antiserum. Phytohaemogglutinin (0.03 ml) or staphylococcal filtrate (0.5 ml) were also added to some other cultures. After 24 hours' incubation 1.0 μc of tritiated thymidine (Radiochemical Centre, Amersham, England) at 4.2 curies/mmole was added when indicated. At 48 hours each culture was transferred to a centrifuge tube, centrifuged at 1000 RPM for 10 minutes, and the supernate discarded.

Smears were prepared from the deposit of cells remaining, and stained with Jenner-Giemsa at pH 5.5 (20). If tritiated thymidine had been added to the culture a second smear from each culture was made. Autoradiographs were made from these smears by layering with Kodak AR 10 stripping film and exposing the film for 5 to 7 days. The film was then developed in Kodak D 19 B developer and the autoradiograph stained with Jenner-Giemsa.

Parallel cultures were usually made to determine DNA synthesis (22) by adding 1.0 ml of cell suspension containing 5×10^6 cells/ml with the identical culture fluid, serum, and stimulant as used for the slide culture. After 24 hours' incubation 0.05 μc of 14C thymidine at

26.0 μc/mmole was added to each culture, and the culture was incubated for a further 24 hours. The cultures were then centrifuged at 1000 RPM for 10 minutes, and washed once in 0.85 per cent NaCl. The button of cells obtained was allowed to dry at 37°C and then dissolved in 1.0 ml hyamine (Nuclear Enterprises Limited, Edinburgh). The hyamine cell solution was transferred to standard counting bottles. Each centrifuge tube was washed twice with a total of 10 ml of phosphore solution (6 gm 2.5 diphenyloxazole (PPO) and 0.12 gm 1.4 bis-(2-(5-phenyloxazoly)-benzene (POPOP) per litre of xylene, kindly supplied by Mr. R. J. Chance, Department of Medical Biochemistry, University of Birmingham) and the washings added to the counting bottle. The amount of radioactivity present in each bottle was determined on

TABLE I

Effect of an Antiallotype As4 Serum on Lymphocytes from an As1,4 Rabbit

Donor cells			Donor serum			Blasts	14C thymidine uptake
Rabbit no.	Allotype	Remarks	Rabbit no.	Allotype	Remarks		
						per cent	*counts/10 min.*
5834	As1,4	Normal	5834	As1,4	Normal	<1	145
5834	As1,4	Normal	5626	As1,3,5,6	Anti-As4	15	3190
5834	As1,4	Normal	5626	As1,3,5,6	Anti-As4 filtered, frozen and thawed	13	2860
5834	As1,4	Normal	5626	As1,3,5,6	Anti-As4 decomplemented	14	2140
5834	As1,4	Normal	5626	As1,3,5,6	Anti-As4 absorbed with As4	<1	307
5834	As1,4	Normal	5671	As1,3,5,6	Normal	<1	201
5834	As1,4	Normal	5314	As1,4	*	<1	253
5627	As1,3,5,6	Anti-As4	5626	As1,3,5,6	Anti-As4	<1	110
5627	As1,3,5,6	Anti-As4	5834	As1,4	Normal	<1	175
5671	As1,3,5,6	Normal	5671	As1,3,5,6	Normal	<1	227
5671	As1,3,5,6	Normal	5626	As1,3,5,6	Anti-As4	<1	210
5671	As1,3,5,6	Normal	5834	As1,4	Normal	<1	204
5314	As1,4	*	5314	As1,4	*	<1	369
5314	As1,4	*	5830	As1,4	Normal	<1	440
5314	As1,4	*	5626	As1,3,5,6	Anti-As4	17	2570

* Immunized with As1,4 γ-globulin and *P. vulgaris*. However, since the γ-globulin allotype of animal 5314 is also As1,4, no anti-As4 antibody was made.

a Packard automatic scintillation counter (Nuclear-Chicago Corporation, Des Plaines, Illinois). The activity was corrected for colour quenching and recorded as disintegrations per 10 minutes.

Agglutination Test.—The ability of antiallotype sera, phytohaemagglutinin (PHAE, and staphylococcal filtrate (SF) to agglutinate As4 lymphocytes was determined by the method of Killman (23). One drop of lymphocyte-rich medium containing 5×10^6 cells/ml was mixed with 1 drop of anti-As4 sera, the mixture incubated at 37°C for 45 minutes, and a drop of the mixture placed on a glass slip. The degree of agglutination was graded microscopically from 0 to +++ (23). In order to test agglutination with PHA and SF, 1 drop of autologous serum was added to 1 drop of cell suspension and then 1 drop of PHA or 4 drops of SF added.

An indirect test for anti-As4 antibody was also performed in the following manner. A culture of As4 lymphocytes was set up using either anti-As4 sera (raised in an As5 animal) or autologous serum. After incubation for 1 hour the cells were washed twice with a total of 6 ml

of medium. The button of cells obtained after the second washing was suspended in 1 drop of strong anti-As5 serum. This anti-As5 suspension was incubated for an additional 30 to 45 minutes and the cells transferred to a microscopic slide. The degree of agglutination was estimated microscopically as described above for the direct agglutination test.

RESULTS

Transformation of As4 Lymphocytes with Anti-As4 Serum.—The results of a representative complete experiment are given in Table I, and the results of over 20 separate experiments using anti-As4 sera prepared in 3 different rabbits (5626, 5627, and 5590) are summarized in Table II. Microscopic analysis of the smears from cultures of normal cells with any serum other than that containing antibody directed against the allotype (As4) present in the donor cells revealed normal small lymphocytes with a well-defined homogeneous nucleus surrounded by a thin rim of blue cytoplasm. However, when the cells from rabbits having As4 γ-globulin allotype were grown in the presence of specific anti-As4–allotype serum, a significant (up to 40 per cent in some cultures) proportion of cells had the typical microscopic appearance of transformed "lymphoblasts" (13, 24). Transitional forms with an appearance intermediate between normal lymphocytes and large "blast" cells were numerous. The large blast cells had a round or oval nucleus with 1 or more prominent nucleoli and a rich basophilic non-granular cytoplasm that usually contained a large clear area (Fig. 1). This effect was essentially unchanged if the antiallotype serum was heated at 56° for 30 minutes, frozen and thawed 1 to 5 times, or passed through a Seitz filter. However, the effect could be removed by absorption of the anti-allotype (As4) serum with serum containing specific As4 allotype. Serum from an As1,4 animal (5314) immunized with *P. vulgaris* and As1,4 γ-globulin in an identical manner to that used for the As1,3,5,6 animals and containing no demonstrable anti-As4 antibody, was not effective in including blast transformation *in vitro*. However, the cells derived from this rabbit were transformed with anti-As4 sera raised in the As1,3,5,6 animals.

TABLE II

Transformation of As4 Lymphocytes with Anti-As4 Sera

Donor cells	Donor serum	No. of determinations	Blasts		14C thymidine uptake (counts/10 min.)	
			Mean	Range	Mean	Range
			per cent	*per cent*		
As1,4	As1,4 Normal	30	<1	<1	308	104–623
As1,4	As1,3,5,6 Normal	12	<1	<1	314	125–635
As1,4	As1,3,5,6 Anti-As4	36	18	2–40	3300	840–13,180

Similar blast cells were produced by the addition of phytohaemagglutinin or staphylococcal filtrate to the culture medium (Figs. 2 through 4) but killed *P. vulgaris* (organisms used as adjuvant in producing the antiallotype serum) or a saline extract of these organisms failed to induce transformation of lymphocytes from normal or immunized rabbits.

Cultures in which blast cells were produced by the addition of specific antiallotype serum, phytohaemagglutinin, or staphylococcal filtrate, all showed a marked increase in 14C thymidine uptake over controls indicating DNA synthesis. Autoradiographs made from lymphocyte cultures after addition of tri-

TABLE III

Effect of Heterologous Antisera to Rabbit γ-Globulin on Rabbit Lymphocytes In Vitro

Serum	Rabbit No. 1		Rabbit No. 2	
	Blasts	14C thymidine uptake	Blasts	14C thymidine uptake
	per cent	*counts/10 min.*	*per cent*	*counts/10 min.*
Autologous	<1	831	<1	677
Antiallotype	37	48,300	29	35,600
Normal sheep	3	967	<1	504
Sheep anti-γ	7	1,496	12	1,754
Normal G.P.	<1	559	<1	578
G.P. anti-γ (1)	<1	1,970	6	2,010
G.P. anti-γ (2)	3	2,360	11	1,480
Goat anti-γ	17	19,550	12	10,065

tiated thymidine revealed diffused nuclear labelling of approximately 33 to 41 per cent of the blast cells formed after addition of SF, 19 to 27 per cent of the blasts formed by PHA stimulation and only 3 to 8 per cent of the blasts stimulated by antiallotype serum. Cells in mitosis could be occasionally identified in the smears obtained from 48 hour cultures with each of these stimulants (Fig. 4).

Effect of Heterologous Anti-Rabbit γ-Globulin Sera on Rabbit Lymphocytes.—Normal goat, guinea pig, and sheep sera supported the growth of rabbit lymphocytes *in vitro* and did not induce significant blast transformation or DNA synthesis. On the other hand, anti-rabbit γ-globulin sera prepared in these animals were capable of stimulating blast transformation and DNA synthesis in degrees comparable to antiallotype serum when added to *in vitro* rabbit lymphocyte cultures (Table III). The histologic appearance of these transformed cells was essentially identical to that of the antiallotype transformed cells described above.

Effect of Pulse Incubation with Anti-As4 Serum (5626) on Lymphoblast Formation from As1,4 Donor Cells.—To determine the length of time for which anti-

body and the appropriate lymphocytes must be in contact in order to induce blast transformation, As1,4 cells were set up in culture with an anti-As4 serum

TABLE IV

Effect of Pulse Incubation with Anti-As4 Serum (5626) on Lymphoblast Formation of As1,4 Donor Cells

Time of incubation with anti-As4 serum*	Blasts	14C thymidine uptake
	per cent	*counts/10 min.*
0 min.	<1	216
15 min.	9	3,201
30 min.	11	3,300
45 min.	21	8,690
60 min.	22	5,590
180 min.	26	12,930
48 hrs.	24	8,830

* The total time of incubation of all cultures was 48 hours. The anti-As4 serum was replaced with normal serum of the same allotype (As1,3,5,6) as the anti-As4 serum at the time indicated.

TABLE V

Effect of Preincubation of Rabbit Lymphocytes with Different Allotype Sera on Transformation In Vitro

Donor cells	Preincubation serum	Time of preincubation	Final serum	Blasts	14C thymidine uptake
				per cent	*counts/10 min.*
As1,4	As1,4	5 min.	As1,3,5,6 (anti-As4)	17	2840
As1,4	As1,4	2 hrs.	As1,3,5,6 (anti-As4)	15	1854
As1,4	As1,4	16 hrs.	As1,3,5,6 (anti-As4)	11	930
As1,4	—	—	As1,4	<1	210
As1,4	As1,6	5 min.	As1,3,5,6 (anti-As4)	8	1446
As1,4	As1,6	2 hrs.	As1,3,5,6 (anti-As4)	12	1210
As1,4	As1,6	16 hrs.	As1,3,5,6 (anti-As4)	7	840
As1,6	—	—	As1,6	<1	156
As1,6	As1,4	5 min.	As1,3,5,6 (anti-As4)	<1	152
As1,6	As1,4	2 hrs.	As1,3,5,6 (anti-As4)	<1	117
As1,6	As1,4	18 hrs.	As1,3,5,6 (anti-As4)	<1	149
As1,6	As1,6	18 hrs.	As1,3,5,6 (anti-As4)	<1	128

(5626). After the intervals indicated in Table IV, the cells were separated from the antiserum by centrifugation and washed twice in medium. The cells from each culture were then resuspended in 1 ml of medium and added to bijou

bottles containing 2.0 ml of medium and 0.5 ml of non-immunized rabbit serum of the same γ-globulin allotype as the anti-As4 serum (As 1,3,5,6). At 48 hours from the original setting up of the cultures the cells were harvested, the degree of transformation recorded, and the 14C thymidine uptake determined. The results are given in Table IV. Incubation with anti-As4 antiserum for only 15 minutes resulted in a significant degree of transformation and DNA synthesis. Incubation for 45 minutes was sufficient to induce the same degree of transformation and DNA synthesis as was found when the anti-As4 serum was in contact with the As4 cells for the entire 48 hours of culture.

TABLE VI

Effect of Dilution on Allotypic Lymphoblast Formation

Serum*	Allotype of donor cells			
	As1,3,4		As1,3,4	
	Blasts	14C thymidine uptake	Blasts	14C thymidine uptake
	per cent	*counts/10 min.*	*per cent*	*counts/10 min.*
Autologous	<1	536	<1	602
5626	25	9,320	9	1945
5626 1:2	37	16,900	10	2021
5626 1:5	33	15,100	4	2335
5626 1:10	12	8,920	1	716
5626 1:20	1	2,450	<1	655
5627	Toxic	13,100	Toxic	4440
5627 1:2	26	14,230	Toxic	1593
5627 1:5	21	3,910	17	2280
5627 1:10	4	1,705	9	1003
5627 1:20	2	1,310	4	636

* Total serum volume 0.5 ml per culture. All dilutions of anti-As4 sera were made with normal rabbit serum with γ-globulin allotype of As1,3,5,6.

Effect of Preincubation of Rabbit Lymphocytes with Different Allotype Serum on Transformation.—To determine whether preincubation of As4 cells in non-As4 serum or of non-As4 cells in As4 serum might alter the reaction of the cells when subsequently cultured *in vitro* in anti-As4 serum a series of 6 experiments exemplified in Table V were performed. Incubation of As4 cells in As1,4 or As1,6 serum for from 5 minutes to 16 hours before washing and culture of the cells in anti-As4 serum failed to alter significantly the transforming capacity of the As4 cells. Incubation of As1,6 cells in As1,4 serum before the addition of anti-As4 serum did not change the non-reactivity of the As1,6 cells to anti-As4 serum. Thus, preincubation of cells of a given allotype with serum of a different allotype does not alter the reactivity of the cells.

Effect of Dilution of Antiallotype Serum on Lymphocyte Transformation.—

To test in part the effect of antibody concentration on transformation, experiments similar to those shown in Table VI were performed. In order to maintain the viability of the cells *in vitro*, it was necessary to keep the serum concentration of the culture system at approximately 20 per cent. Dilutions of the anti-As4 serum were made with normal rabbit serum of the same allotype (As1,3,5,6) as the anti-As4 serum.

TABLE VII

Absorption of Anti-As4 Antibody Activity by As4 Lymphocytes

No. of lymphocytes per culture ($\times 10^6$)	Duration of each culture in antiserum	No. of culture passages*	Degree of transformation of final lymphocyte culture*		Haemagglutination titer of supernate after final culture (dilution)
			Blasts	14C thymidine uptake	
	hrs.		*per cent*	*counts/10 min.*	
5	1	1	16	3195	1:400
5	1	2	11	2220	1:40
5	1	3	2	892	1:5
5	1	4	<1	588	Negative
5	2	1	17	5107	1:400
5	4	1	14	2475	1:200
5	24	1	12	2910	1:400
20	1	1	15	8949	Negative
5‡	1	4	—	—	Negative
20‡	1	1	—	—	Negative
20§	1	1	<1	389	1:2000
5	48	1	18	3463	1:400

* The total time of incubation of all cultures was 48 hours. Medium containing normal rabbit serum of the same allotype as the antiserum (As1,3,5,6) was added to each culture if the antiserum containing medium was removed before 48 hours.

‡ Heat-killed cells.

§ Non-As4 lymphocytes.

The degree of lymphocyte transformation and amount of DNA synthesis were directly related to the amount of antibody present. At high concentration, inhibition or toxicity was noted with strong antisera. This finding is consistent with those of Gräsbeck *et al.* (25) using rabbit antisera to human leucocytes.

Absorption of Anti-As4 Serum with As4 Cells.—To determine if antiallotype activity is removed by lymphocyte culture the experimental plan as depicted in Table VII was followed. Four As4 lymphocyte-anti-As4 serum (5626) cultures were set up in the usual manner. At the end of 1 hour, the cultures were centrifuged and the supernate antiserum-containing-medium of 1 culture was saved and frozen for further analysis, while the supernates from the remaining 3 cultures were each added to separate bijou bottles with 0.25 ml leucocyte-medium suspension containing 5×10^6 white cells. After an additional 1 hour

incubation, these 3 cultures were centrifuged, 1 supernate saved, and the other 2 supernates added to additional leucocyte suspensions. At the end of another hour's incubation 1 of these supernates was saved, while the other underwent an additional 1 hour incubation with As4 lymphocytes. In addition, the supernates of cultures of 5×10^6 lymphocytes for 2 hours, 4 hours, and 24 hours were obtained by centrifugation and saved. Supernates were also obtained after 1 hour's culture with 20×10^6 As4 lymphocytes and with 20×10^6 heat-killed (48°C for 30 minutes) As4 lymphocytes; after 4 1-hour passages with 5×10^6 heat-killed As4 lymphocytes, and after 1 hour's culture with 20×10^6 As1,3,5,6, lymphocytes. All the supernates obtained were tested for anti-As4 activity by agglutination of sheep erythrocytes coated with As1,4 γ-globulin.

TABLE VIII

Effect of Mixing of Cells of Different Allotypes (Experiment 21)

Normal donor cells—I	Volume	Donor cells II	Volume	Remarks	Donor serum	Blasts	14C thymidine uptake
						per cent	*counts/10 min.*
As1,4	0.5	As1,3,5,6 Anti-As4	0.5		As1,3,5,6	<1	420
As1,4	1.0	As1,3,5,6 Anti-As4	1.0	II killed	As1,3,5,6	<1	276
As1,4	1.0	As1,3,5,6 Anti-As4	1.0	I killed	As1,3,5,6	<1	392
As1,4	0.5	As1,3,5,6 Normal	0.5		As1,3,5,6	<1	209
As1,4	1.0	—	—		As1,3,5,6	<1	357
As1,4	1.0	—	—		As1,3,5,6 Anti-As4	9	1690

After removal of the antibody-medium supernate, the viable cells remaining in the bottom of each culture bottle were cultured for a total of 48 hours from the initial addition of antiserum by adding 3 ml of medium and 0.5 ml of normal rabbit serum of the same allotype as the antiserum (As1,3,5,6). These cultures were processed as described above for determination of degree of blast transformation and DNA synthesis.

The results of one experiment are given in Table VII. All of the anti-As4 activity as measured by transforming or haemagglutinating capacity was absorbed by incubation for 1 hour with 20×10^6 viable or heat-killed As4 lymphocytes. A progressive loss of activity was recorded after successive passages of antiserum containing medium with 5×10^6 As4 lymphocytes, and total loss recorded after the fourth passage. Incubation of antisera with 5×10^6 lymphocytes for 2, 4, 24, or 48 hours did not result in greater loss of activity than incubation with the same number of cells for 1 hour. Cultures containing 20×10^6 non-As4 lymphocytes did not remove any anti-As4 activity.

It should be noted here that removal of the anti-As4 activity is probably

not due to small amounts of As4 serum in the lymphocytes preparations as all cells were washed once in medium and then diluted in medium so that the calculated amount of original serum remaining a standard lymphocyte culture (5×10^6 cells) would be equivalent to 0.001 ml. This amount of As4 serum added to 0.5 ml anti-As4 serum has no effect on its subsequent antibody activity. In addition, absorption of 100 ml of anti-As4 serum with 0.5 ml packed, washed erythrocytes from an As4 donor does not alter the activity of the antiserum.

Effect of Mixing of Cells of Different Allotypes.—Because of recent reports of "blast" cell formation when lymphocytes of 2 different individuals were mixed

TABLE IX

Effect of 7S and 19S Fractions of Anti-As4 Serum on As4 Lymphocytes

Serum or fraction	Haemagglutination titer (against As4 coated cells)	Allotype of donor cells					
		As1,4		As1,2,4,6		As1,4,6	
		Blasts	14C thymidine uptake	Blasts	14C thymidine uptake	Blasts	14C thymidine uptake
		per cent	*counts/10 min.*	*per cent*	*counts/10 min.*	*per cent*	*counts/10 min.*
Anti-As4	1:1000	41	9,960	24	10,600	7	2350
7S*	1:1250	49	14,360	—	—	Toxic	2761
19S*	<1:10	0	416	—	—	0	387
7S‡	1:1000	—	—	11	2,380	6	1015
19S‡	<1:10	—	—	0	133	0	154

* Ultracentrifuge fractions.

‡ Sephadex G-200 fractions.

in vitro (14–16) the effect of mixing of cells from rabbits of different allotypes was determined. Table VIII gives the results of a typical experiment, of which the basic plan was repeated 3 times. Mixtures of cells from different rabbits, even if 1 donor was immunized against the allotype of the other donor, never resulted in blast cell transformation or increased 14C thymidine uptake over control cultures.

Effect of 7S and 19S Fractions of Anti-As4 Serum on As4 Cells.—To determine the type of antibody involved in transformation, an anti-As4 serum (5626) was separated by ultracentrifugation (kindly performed by Dr. Normansell) and by gel filtration on sephadex G-200 (kindly performed by Dr. David S. Rowe). The fractions obtained were dialysed against 0.85 per cent NaCl solution and each fraction concentrated by ultrafiltration to approximately the volume of the starting serum. The fractions were sterilized by Seitz filtration and added to lymphocyte cultures in 0.5 ml aliquots. In order to maintain the concentra-

tion of normal serum at approximately 20 per cent 0.5 ml of normal rabbit serum of the same allotype as the anti-As4 serum (As1,3,5,6) was also added.

The results are given in Table IX and indicate that most, if not all, of the transforming capacity of the anti-allotype serum is found in the 7S fraction. Although none of the 19S fractions produced lymphoblast transformation or significant DNA synthesis, trace amounts of antibody activity in the 19S fractions cannot be entirely ruled out.

Absence of Agglutination of As4 Lymphocytes with Anti-As4 Serum.—The anti-As4 sera, normal rabbit serum (5672), autologous sera and SF are essentially non-agglutinating. PHA was always strongly agglutinating. The presence of non-agglutinating anti-As4 antibody on the surface of As4 cells was tested

TABLE X

Effect of As4 Serum on Lymphocytes from Rabbits Immunized against As4 Allotype

Donor cells	Donor serum	No. of cultures with per cent blasts			No. of cultures with 14C thymidine uptake (counts/10 min.)		
		<1	1–2	>2	100–500	500–750	>750
As1,3,5,6 immunized with As4 allotype	As1,3,5,6	23	0	0	17	6	0
As1,3,5,6 immunized with As4 allotype	As1,4	20	2	0	12	5	5

for by superagglutination with antibody to an allotype of the anti-As4 γ-globulin (As5). Agglutination was not demonstrable by this technique.

Effect of As4 Serum of Lymphocytes From Rabbits Immunized to As4 Allotype.—In most cases culture of lymphocytes from rabbits immunized to As4 allotype in As4 serum resulted in no blast cell formation and in 14C thymidine uptake not significantly different from controls (Table X). However, in a few cases a definitely increased 14C thymidine uptake was noted (up to 4790 disintegration/10 minutes). This is consistent with the observations of Dutton and Eady that increased DNA synthesis is observed in rabbit spleen suspensions *in vitro* when suspensions from immunized animals are cultured in the presence of the immunizing antigen (22). Further studies in our laboratory using heterologous antigen in rabbits have failed to demonstrate convincingly any stimulation of *in vitro* DNA synthesis in peripheral lymphocytes when cultured with antigen (26).

DISCUSSION

Rabbit leucocytes cultured *in vitro* are not only non-specifically stimulated to form "blast" cells and to undergo mitosis by phytohaemagglutinin and

staphylococcal filtrate, agents known to be active on peripheral lymphocytes of human origin (13, 20) and recently shown to stimulate peripheral lymphocytes obtained from laboratory animals (26) but are also transformed when grown in the presence of specific anti-allotype serum. This allotype effect only occurs when the donor cells are obtained from an animal of a given allotype and are grown in an antiserum prepared against the allotype present in the donor of the cells. It is dependent upon the amount of antibody present in the cell cultures, is not dependent upon complement, and does not occur with mixtures of viable cells. Contact between the appropriate lymphocytes and anti-allotype serum for as little as 15 minutes results in a significant induction of blast transformation, indicating that the action of the antisera is almost immediate. This is consistent with the effect of PHA on human cells (27).

The stimulation of leucocytes from a rabbit with a given γ-globulin allotype by specific antiallotype serum indicates that the cells of the donor animal may be identified in the absence of donor serum. Previous identification of γ-globulin allotypes has depended upon the presence of measurable amounts of serum γ-globulin containing the given allotype (17). In the present experiments, the capability of donor cells to transform was not significantly altered by preincubation up to 18 hours with serum that did not contain the allotype. Also, preincubation in As4 serum of cells from a rabbit not having the As4 allotype did not alter the inability of the non-As4 cells to transform in the presence of anti-As4 antibody. Thus, the identification of allotypic specificity by lymphoblast transformation appears to be inherent in the cell that is transformed and not dependent upon the presence of free γ-globulin or of γ-globulin non-specifically bound on the cell surface. However, antigenic determinants of the specific γ-globulin allotype must presumably be present on or in the cell in order for the antiallotype serum to be effective. Allotype-antiallotype systems are one of the most specific antigen-antibody reactions known, and it is hardly conceivable that the antiallotype serum could be effective other than in a reaction with allotype antigenic determinants. Several possible explanations for the allotypic recognition of lymphocytes include: (*a*), that serum γ-globulin is irreversibly fixed on or in the lymphocytes and can react with the antiallotype serum to induce the necessary cellular changes. (*b*), that the lymphocytes manufacture γ-globulin *in vitro* and enough of this γ-globulin is present to be identified by antibody, or (*c*), that genetically determined protein structures are permanently present on or in the cell and these structures carry the allotype antigenic determinants of γ-globulin. However, the presence of the As4 allotype in normal or transformed As4 cells could not be demonstrated using specific fluorescent anti-As4 antibody (28).

The capacity of the antiallotype-allotype reaction to produce lymphoblast transformation is probably not due to a specific property of the rabbit antibody molecule as contrasted to the antibodies from other species or to a specific

manifestation of the allotype-antiallotype reaction, as heterologous antisera to rabbit γ-globulin are also capable of inducing blast transformation and DNA synthesis in rabbit lymphocyte cultures. However, results to be published in a following paper indicate that allotypic stimulation is specific to particular determinants in the γ-globulin molecule in so far as the effects of stimulating the appropriate lymphocyte cultures with mixtures of antisera to 2 different allotypes are additive.

The "blast" cells found after stimulation with phytohaemagglutinin, staphylococcal filtrate, specific antiallotype serum or heterologous antisera to rabbit γ-globulin are most likely derived from the circulating small lymphocytes. Cultures of human thoracic duct lymphocytes (29) and purified lymphocyte preparations (30) also incriminate the small lymphocyte as the originator of the blast cell when cultured with certain stimulants, and the histochemical staining characteristics of human blast cells indicate a lymphocytic origin (31). Over 90 per cent of the white cells in the starting leucocyte preparations from the rabbits used in the preparations described here were lymphocytes. The few polymorphonuclear leucocytes present were largely degenerated after 24 hours *in vitro* and the occasional macrophages present were easily identified. In stimulated cultures transitional forms could be identified between the lymphocytes and "blast" cells. Although origin from a cell other than the small lymphocyte cannot be entirely eliminated, the evidence overwhelmingly suggests that these stimulated blast cells are derived from the small lymphocyte.

Some workers have noted a morphologic resemblance between some stimulated blast cells and immature plasma cells (32, 33) and a differentiation of transformed cells to plasma cells has been postulated (32, 33). The cytologic appearance of a "blast" cell is consistent with a hemocytoblast or "transitional cell" of Fagraeus (34) with a large circular leptochromatic nucleus and prominent nucleoli, a rim of pyroninophilic cytoplasm, and a large clear area in the cytoplasm that extends from the nuclear membrane almost to the edge of the cytoplasmic membrane. Many "transitional forms" between small lymphocytes and large blast cells may be present, and these cells may be confused with immature plasma cells. However, the nuclei of transformed lymphocytes never demonstrate the typical cartwheel chromatin pattern of the plasma cell. In fact, no mature plasma cells have ever been seen in any of the *in vitro* cultures of peripheral lymphocytes. Thus, it is unlikely that the transformed cells are differentiating into plasma cells. Cells in mitosis, or large blast cells present in smears from stimulated cultures, are usually about 5 times larger than the small lymphocytes. Rarely one may come across 2 daughter cells, identifiable by the presence of visible chromosomes and the close approximation of the cells. These cells are approximately the same size as the small lymphocytes. It appears, then, that blast transformation is a manifestation of preparation for mitosis and that the end result of blast transformation is mitosis. This inter-

pretation is supported by electron microscopic observations of PHA stimulated human blast cells which reveal an altered organization of cytoplasm associated with cell division (35) and a relative absence of the characteristic endoplasmic reticulum of plasma cells (35–37).

The morphologic similarity of blast cells formed from rabbit lymphocytes by stimulation with phytohaemagglutinin, with staphylococcal filtrate, with anti-γ-globulin allotype serum, and with heterologous antisera to γ-globulin may be the manifestation of a similar mechanism in all cases or may simply illustrate the pathologic principle that a given tissue has a limited potential morphologic ability to respond to different stimuli. At least some of the cells transformed by the 4 different stimulants must arise from the same population of lymphocytes because in some cultures up to 90 per cent of the cells were stimulated to undergo blast transformation with PHA, up to 70 per cent with SF, up to 40 per cent with antiallotype serum, and up to 20 per cent with heterologous antisera to rabbit γ-globulin.

The potent leucoagglutinating properties of PHA (13, 24) and rabbit anti-human leucocyte serum (25), and the inability to separate the leucoagglutinating from the mitogenic activity (38–40), led to the conclusion that agglutination was necessary to produce blast transformation (38–40). However, lymphocyte agglutination is not a property of antiallotype sera nor of staphylococcal filtrate (20). Therefore it must be concluded that agglutination is not necessary to produce blast transformation with these systems. In addition, the failure to demonstrate the presence of antiallotype antibody on the surface of the appropriate lymphocyte by indirect agglutination tests indicates that the induction of transformation may not be a surface phenomenom and that recognition takes place within the appropriate cells.

The removal of anti-As4 antibody activity after culture with As4 lymphocytes indicate either; (*a*), that anti-As4 antibody is irreversably bound to some structure in the cell, or (*b*), that the antibody is destroyed or inactivated after reacting with the As4 lymphocytes. The finding that killed lymphocytes of the appropriate allotype will also remove antibody activity is evidence against, but does not rule out, the second possibility. The absorption of antibody activity by As4 lymphocytes is not due to non-specific cytophilic binding because antibody activity is not removed by non-As4 lymphocytes. Further studies on the metabolic properties of transformed rabbit lymphocytes and on the mechanism of action of the antiallotype serum are currently underway.

SUMMARY

Rabbit lymphocytes may be stimulated *in vitro* with specific antiallotype sera to transform into "blast" cells and to synthesize DNA. This transformation only occurs when the donor cells are obtained from a rabbit having a given γ-globulin allotype (As4) and these cells are cultured in the presence of an

antiserum prepared against the given allotype (As4). Heterologous (sheep, goat, and guinea pig) anti-rabbit γ-globulin sera also induce significant blast transformation and DNA synthesis in rabbit lymphocytes. Allotypic transformation and DNA synthesis are due to 7S antiallotype antibodies and do not require complement. The degree of transformation and rate of DNA synthesis is related to the concentration of antibody. Incubation of the appropriate cells with the antiallotype antibody for as short a time as 15 minutes results in a significant degree of "blast" transformation, indicating that the recognition of the antiallotype specificity in the cells and stimulation of the cellular changes leading to eventual transformation is rapid. The activity of the antiallotype sera as measured by transforming or haemagglutinating capacity, may be absorbed by lymphocytes of the appropriate allotype, but is not absorbed by lymphocytes from a donor rabbit not having the allotype to which the antiserum is directed. Transformation does not occur with mixtures of lymphocytes from different rabbits even if 1 donor is immunized against an allotype present in the other donor.

Peripheral rabbit lymphocytes can also be induced to undergo "blast transformation" *in vitro* by phytohaemagglutinin and staphylococcal filtrate. The lack of demonstrable leucoagglutinins in staphylococcal filtrate and antiallotype serum indicates that agglutination is not a necessary prerequisite to the induction of blast transformation.

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BIBLIOGRAPHY

1. Trowell, O. A., The lymphocyte, *Internat. Rev. Cytol.*, 1958, **7**, 235.
2. Gowans, J. L., and Knight, E. J., The route of re-circulation of lymphocytes in the rat, *Proc. Royal Soc. London, Series B*, 1964, **159**, 257.
3. Landy, M., Sanderson, R. P., Bernstein, M. T., and Jackson, A. L., Antibody production by leucocytes in peripheral blood, *Nature*, 1964, **204**, 1320.
4. Baney, R. N., Vazques, J., and Dixon, F. J., Cellular proliferation in relation to antibody synthesis, *Proc. Soc. Exp. Biol. and Med.*, 1962, **109**, 1.
5. Gowans, J. L., McGregor, D. D., Cowen, D. M., and Ford, C. E., Initiation of immune responses by small lymphocytes, *Nature*, 1962, **196**, 651.
6. Nossal, G. J. V., and Mäkelä, O., Autoradiographic studies on the immune response I. The kinetics of plasma cell proliferation, *J. Exp. Med.*, 1962, **115**, 209.
7. Gowans, J. L., The fate of parental strain small lymphocytes in F_1 hybrid rats, Ann. New York Acad. Sc., 1962, **99**, 432.
8. Billingham, R. E., Defendi, V., Silvers, W. K., and Steinmuller, D., Quantitative studies on the induction of tolerance of skin homografts and on runt disease in neonatal rats, *J. Nat. Cancer Inst.*, 1962, **28**, 365.
9. Miller, J. F. A. P., The thymus and the development of immunologic responsiveness, *Science*, 1964, **144**, 1544.

10. East, J., and Parrott, D. M. V., Prevention of wasting in mice thymectomized at birth and their subsequent rejection of allogeneic leukemic cells, *J. Nat. Cancer Inst.*, 1964, **33**, 673.
11. Gowans, J. L., and McGregor, D. D., The origin of antibody forming cells, *Immunopathol. Internat. Symp. 3rd, La Jolla, California*, 1963, 89.
12. Delorme, E. J., Recovery from lethal irradiation in rats after intravenous administration of isogenic lymphocytes, *Lancet*, 1961, **2**, 855.
13. Robbins, J. H., Tissue culture studies of the human lymphocyte, *Science*, 1964, **146**, 1648.
14. Bain, B., Vas, M., and Lowenstein, L., The development of large immature mononuclear cells in mixed leukocyte cultures, *Blood*, 1964, **23**, 108.
15. Bain, B., and Lowenstein, L., Genetic studies on the mixed leukocyte reaction, *Science*, 1964, **145**, 1315.
16. Rubin, A. L., Stenzel, K. H., Hirschhorn, K., and Bach, K., Histocompatibility and immunologic competence in renal homotransplantation, *Science*, 1964, **143**, 815.
17. Dray, S., Dubiski, S., Kelus, A., Lennox, E. S., and Oudin, J., A notation for allotypy, *Nature*, 1962, **195**, 785.
18. Gell, P. G. H., and Kelus, A., unpublished data.
19. Dubiski, S., Dudziak, Z., Skalba, D., and Dubiska, A., Serum groups in rabbits, *Immunology*, 1959, **2**, 84.
20. Ling, N. R., Spicer, E., James, K., and Williamson, N., The activation of human peripheral lymphocytes by products of staphlococci, *Brit. J. Haematol.*, in press.
21. Coulson, A. S., and Chalmers, D. G., Separation of viable lymphocytes from human blood, *Lancet*, 1964, **1**, 468.
22. Dutton, R. W., and Eady, J., An *in vitro* system for the study of the mechanism of antigenic stimulation in the secondary response, *Immunology*, 1964, **7**, 40.
23. Killmann, S. Å., Studies on the leucocyte-agglutination, *Acta Path. et Microbiol. Scand.*, 1959, **45**, 17.
24. Nowell, P. C., Phytohemagglutinin: an initiator of mitosis in cultures of normal human leukocytes, *Cancer Research*, 1960, **20**, 462.
25. Gräsbeck, R., Nordman, C. T., and de la Chapelle, A., The leucocyte mitogenic effect of serum from rabbits immunized with human leucocytes, *Acta Med. Scand.*, 1964, suppl. 412, 39.
26. Knight, S., Ling, N. R., Sell, S., and Oxnard, C. E., Transformation *in vitro* of the peripheral lymphocytes of some laboratory animals, *Immunology*, in press.
27. Newsome, J., *In vitro* production of antibody lymphocytes, *Lancet*, 1963, **2**, 91.
28. Sell, S., and Gell, P. G. H., unpublished data.
29. Lindahl-Kiessling, K., Werner, B., and Böök, J. A., Short term cultivation of human thoracic duct lymphocytes with phaseolus vulgaris extract, Presented at the meeting of the Medical Society of Uppsala, December 18, 1963.
30. Carstairs, K., The human small lymphocyte: Its possible pluripotential quality, *Lancet*, 1962, **1**, 829.
31. Quaglino, D., Hayhoe, F. G. J., and Flemans, R. J., Cytochemical observations on the effect of phytohaemagglutinin in short-term tissue cultures, *Nature*, 1962, **196**, 338.

32. Bach, F., and Hirschhorn, K., γ-globulin production by human lymphocytes *in vitro*, *Exp. Cell Research*, 1963, **32**, 592.
33. Elves, M. W., Roath, S., Taylor, G., and Israels, M. C. G., The *in vitro* production of antibody lymphocytes, *Lancet*, 1963, **1**, 1292.
34. Fagraeus, A., Antibody production in relation to the development of plasma cells, *Acta Med. Scand.*, 1948, suppl. 204, 3.
35. Inman, D. R., and Cooper, E. H., Electron microscopy of human lymphocytes stimulated by phytohaemagglutinin, *J. Cell. Biol.*, 1963, **19**, 441.
36. Tanaka, Y., Epstein, L. B., Brecher, G., and Stohlman, F., Jr., Transformation of lymphocytes in cultures of human peripheral blood, *Blood*, 1964, **22**, 614.
37. Elves, M. W., Gough, J., Chapman, J. A., and Israels, M. C. G., Electron microscopy studies of lymphocytes. Transformation under the influence of phytohaemagglutinin, *Lancet*, 1964, **1**, 306.
38. Rigas, D. A., and Johnson, E. A., Studies of the phytohemagglutinin of *Phaseolus vulgaris* and its mitogenicity, *Ann. New York Acad. Sc.*, 1964, **113**, 800.
39. Nordman, C. T., de la Chapelle, A., and Gräsbeck, R., The interrelations of erythroagglutinating, leucoagglutinating and leucocyte-mitogenic activities in *Phaseolus vulgaris* phytohaemagglutinin, *Acta Med. Scand.*, 1964, suppl. 412, 49.
40. Kolodny, R. L., and Hirschhorn, K., Properties of phytohaemagglutinin, *Nature*, 1964, **201**, 715.

EXPLANATION OF PLATE 28

FIGS. 1 to 4. Transformed lymphocytes produced *in vitro* after stimulation with different agents. (Jenner-Giemsa, × 1000, enlarged × 4).

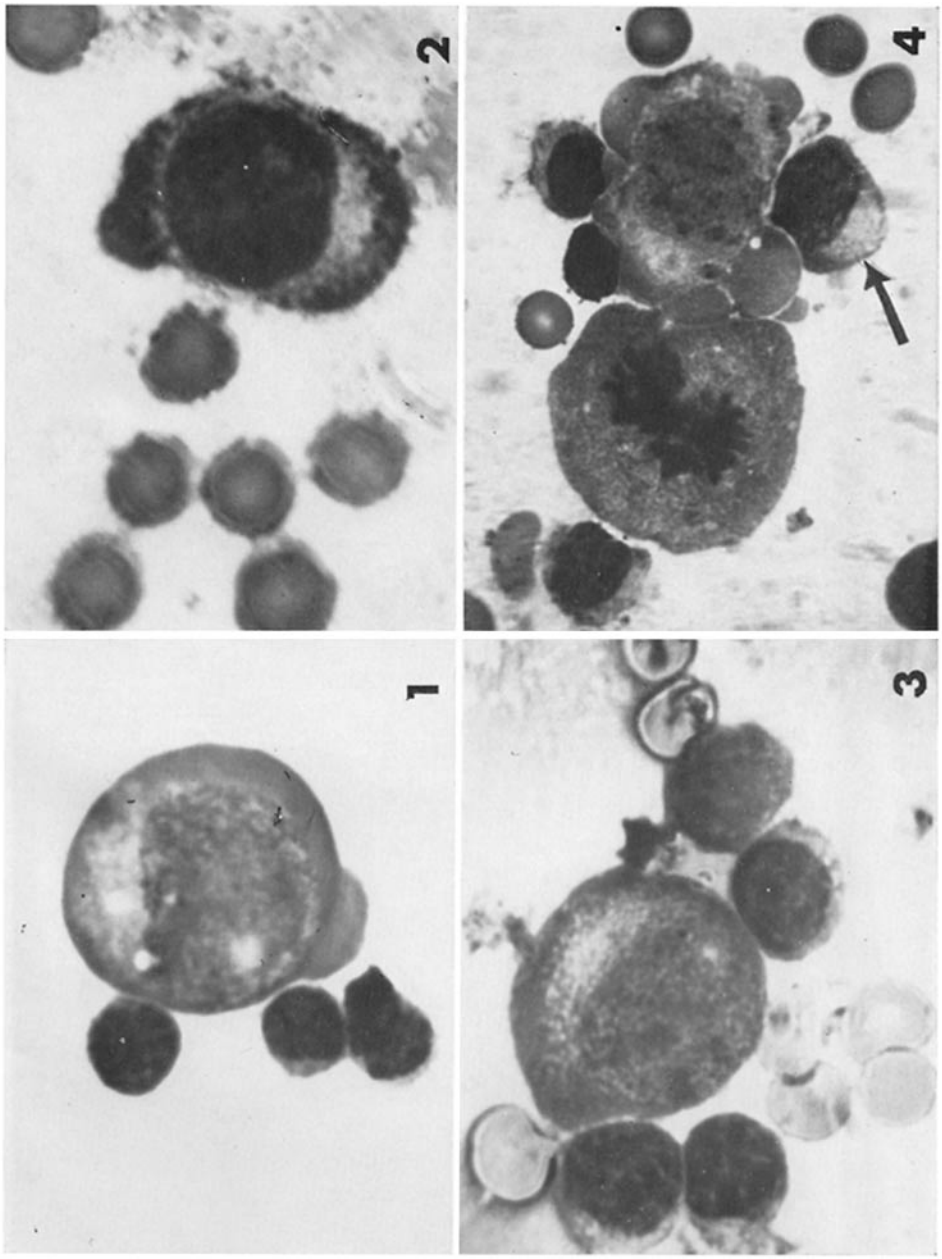
Normal small lymphocytes or erythrocytes are included in each figure for comparison. Note the agglutination of cells after treatment with phytohaemagglutinin. Many transitional forms (indicated by arrow in Fig. 4) may be confused with immature plasma cells. Occasional mitotic figures (Fig 4) are formed after treatment with each stimulant.

FIG. 1. Antiallotype serum.

FIG. 2. Staphylococcal filtrate.

FIG. 3. Phytohaemagglutinin.

FIG. 4. Phytohaemagglutinin.



(Sell and Gell: Rabbit lymphocytes *in vitro*. I)