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Immunoglobulin Determinants on the Lymphocytes of Normal Rabbits

I. DEMONSTRATION BY THE MIXED ANTIGLOBULIN REACTION OF DETERMINANTS RECOGNIZED BY ANTI-γ, ANTI-μ, ANTI-Fab AND ANTI-ALLOTYPE SERA, ANTI-As4 AND ANTI-As6

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Summary. Immunoglobulin determinants can be shown on the membrane of circulating lymphocytes of the rabbit by the mixed antiglobulin reaction. An enumeration was made of lymphocytes reacting specifically with anti-γ, anti-μ, anti-Fab and anti-L chain sera. The L chain allotypic determinants As4 and As6 were also shown and reacting cells enumerated. There was a marked dose-dependence between the concentration of the antiglobulin reagent used to treat the lymphocyte suspension and the number of lymphocytes reacting to form rosettes.

The experiments reported at this stage bear no direct evidence on the question as to whether the μ- and γ-determinants, or more than one L chain allotypic determinant can exist on the one cell.

Of three rabbits with 26, 47, and 61 per cent IgG-reacting lymphocytes in the circulation, only one had 1 per cent reacting cells in the thymus while the other two had less than 1 per cent. No μ-reacting cells were detectable in the thymuses of the two rabbits tested.

INTRODUCTION

There is increasing evidence in the rabbit that lymphocytes, including those in the peripheral blood, show on their surface antigenic determinants identical with those of serum immunoglobulins. This seems to hold for a high percentage of peripheral blood lymphocytes (PBL) judging from the transforming activity of anti-allotypic and heterologous anti-immunoglobulin antisera (Sell and Gell, 1965a; Sell, 1967a, b). Though evidence was produced that such determinants were the product of the cell (Sell and Gell, 1965b) and not adsorbed from the ambient plasma, it was not wholly excluded that at times some of the cells transformed may be secondarily affected by mitogenic substances released from the cells primarily and specifically affected by the antisera. By careful quantitation these authors (Sell and Gell, to be published) concluded tentatively that it is possible for allotypically heterozygous cells to produce and manifest both alleles simultaneously: that is that the phenomenon of allelic exclusion demonstrable with plasma cells (Pernis, Chiappino, Kelus and Gell, 1965) does not hold for 'normal' PBL.

Direct evidence for the presence of immunoglobulin (Ig) determinants on rabbit lymphocytes should be obtainable by means of the mixed antiglobulin reaction (see Coombs and Gell, 1968), especially so since it was found that lymphocytes were not themselves agglutinated by anti-Ig antisera (Sell and Gell, 1965a). In the present investigation, lymphocytes treated with anti-Ig antisera (including anti-allotypic antisera) are found to form rosettes with immunoglobulin-carrying red cells, presumably by means of free valencies (see Figs. 1 and 2). This report describes the procedure for carrying out this reaction and preliminary results of its use.

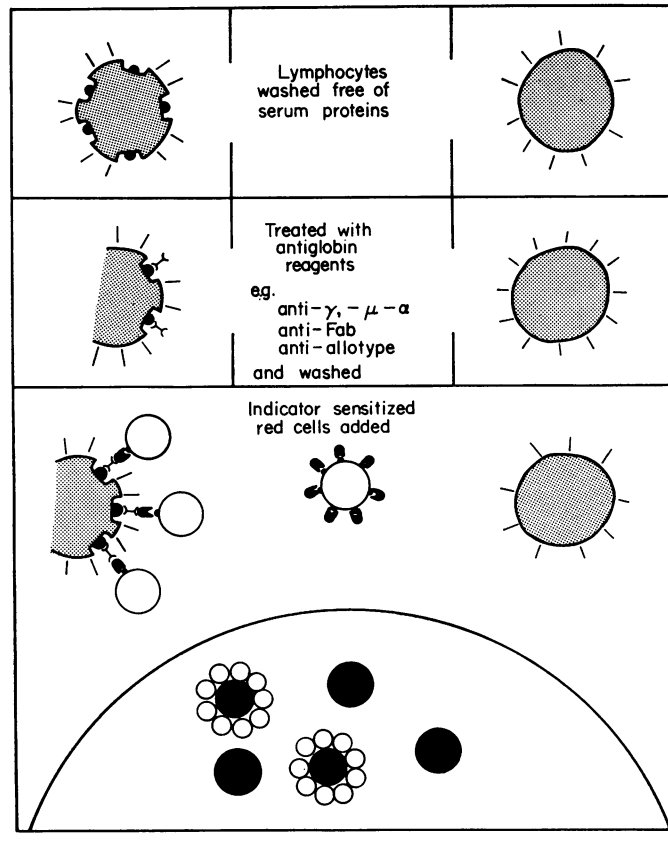

FIG. 1. Diagrammatic representation of the mixed antiglobulin reaction to show immunoglobulin determinants on the cell membrane of the lymphocyte.

MATERIALS AND METHODS

Preparation of lymphocytes for preservation and subsequent testing

Rabbits were bled 23 ml from the ear into a tube and defibrinated with a rotating U-shaped glass rod. Ten millilitres of blood were transferred to each of two Universal Bottles containing 3·5 ml 1 per cent methyl cellulose (in saline) plus 150 mg Carbonyl Iron all previously warmed at 37°. The bottles were well mixed and then rotated at 37° on a Matburn Rotator. The blood was then transferred to pre-warmed siliconed 12×1·5 cm test tubes placed in an upright position in a 37° water-bath. The blood–air interface was

under the water level to avoid convection currents. After 30–40 minutes the leucocyte-rich supernatant serum was taken off and pooled in another test tube. The phagocytic leucocytes and surplus iron were drawn to the bottom of the tube by passing through the jaws of a powerful magnet (Eclipse Major magnet, Catalogue No. 862); the deposit was discarded.

The suspension was then distributed into 5 × 0·8 cm siliconed glass tubes in 1-ml amounts and centrifuged in the cold at 200 ***g*** maximum for 8 minutes, required because of the viscosity of the methyl cellulose. The deposited cells were washed in saline, recentrifuged and then washed again. The cells deposited finally in each tube were resuspended in two drops of 20 per cent glycerol in saline, these were pooled and diluted in the glycerol saline to 5 ml and finally adjusted to give a count of 3000/mm^3 and placed in 1-ml amounts in 2-ml bottles (Trident) over liquid nitrogen in a Linde vacuum container. At this stage there were still contaminating red cells in the suspension.

In recovering the lymphocytes, a bottle was thawed quickly at 37°, 0·5 ml saline was added and 0·75 ml centrifuged in each of two tubes. Saline was used to wash twice the deposited lymphocytes (0·75 ml/wash). During this treatment the red cells lysed and were removed in the supernatant. The lymphocytes were then resuspended in diluent containing 0·2 per cent bovine plasma albumin (BPA) and adjusted to give a suspension of approximately 3000 lymphocytes/mm^3. The suspension was then ready for testing.

Antiglobulin reagents and specific inhibitors

(i) *Sheep* 207/5 *anti-whole rabbit serum.* The sheep was injected with 4 ml rabbit serum emulsified with 4 ml Freund's complete adjuvant. The sheep received four such injections at 3 monthly intervals and was bled 1 month after the fourth injection.

(ii) *Goat A*58/12 *anti-rabbit IgG.* The goat received multiple injections of rabbit IgG purified by DEAE-chromatography. Each injection consisted of 20–30 mg IgG in Freund's complete adjuvant. The serum used was the twelfth bleed.

(iii) *Sheep C*17/111*F anti-rabbit IgG.* This was raised by a similar procedure to (ii).

(iv) *Sheep M*33/3 *anti-rabbit papain Fab.* Papain Fab was prepared from pure IgG. The sheep was injected with 7 mg in Freund's complete adjuvant and bled 6 weeks later.

(v) *Sheep W*431 *anti-rabbit* μ. Rabbit IgM was prepared by the method of Chaplin, Cohen and Press (1965). Sheep W431 was injected with 10 mg in Freund's complete adjuvant and bled 1 month later.

To make this anti-IgM μ-specific, 10 ml was absorbed with 10 mg rabbit IgG (Mann laboratories). A precipitate was obtained which was spun off and after adding another 10 mg no precipitate was obtained after hard centrifugation.

(vi) *Goat A*58 *anti-*γ. To 5 ml of Goat A58 anti-IgG was added 5 mg papain Fab prepared from rabbit IgG. After removal of the precipitate, another 3 mg Fab was added, when a slight precipitate formed. After removal of this no further precipitate formed on adding a further 3 mg Fab.

On immunoelectrophoresis only a single γFc line was obtained.

(vii) *Anti-allotype sera.* Anti-As4. R75/13/P (a1a2b5b6) and anti-As6 R79/53/III (a1a3b4b5).

These were raised by standard methods as described by Dubiski, Dudziak and Skalba (1959), using rabbit anti-*Proteus* antibodies coated onto a suspension of *Proteus vulgaris* OX19.

Before use in mixed antiglobulin tests the antiglobulin sera were heat-inactivated at 56° for ½ hour and absorbed with six-times washed rabbit red cells if the sera were not rabbit and with six-times washed sheep red cells if the sera were not sheep.

The antiglobulin activity and specificity were checked in anti-globulin tests on sheep red cells sensitized with appropriate rabbit antibody.

Anti-sheep red cell sera

(i) *IgG anti-sheep cell antibody.* This was a DEAE-exclusion fraction of a rabbit anti-Forssman serum prepared by injecting a rabbit with an alcoholic extract of horse kidney in Freund's complete adjuvant.

(ii) *IgM anti-sheep cell antibody.* This was a Sephadex G-200 exclusion fraction of a rabbit anti-Forssman serum also prepared by injecting a rabbit with an alcoholic extract of horse kidney in Freund's complete adjuvant.

(iii) *As/4 and As/6 sheep-cell antibodies.* R 81/04/II (As4) and R 78/64/II (As6).

These were raised by intravenous injections of washed sheep red cells.

Diluent

Tris–citrate–Hanks's solution containing 0·2 per cent bovine plasma albumin.

Mixed antiglobulin reaction on rabbit lymphocytes

(i) *Preparation of sensitized indicator sheep red cell suspensions.* The dilution of the rabbit anti-sheep cell reagent used to sensitize the sheep red cell suspension was determined by a previous antiglobulin test. A dilution was chosen which failed to give direct agglutination but sensitized the cells for subsequent detection with antiglobulin.

A 2 per cent red cell suspension was incubated with this selected dilution. After three washes the sensitized red cells were resuspended to 0·4 per cent in diluent containing 0·2 per cent BPA.

(ii) *Stained slides.* A small drop of a fresh mixture of equal parts 1 per cent toluidine blue in methanol and methanol was placed on a series of siliconed 3 × 1 in. glass slides. These were dried in a 37° dry incubator and, once dry, left at room temperature. Before adding the tested lymphocyte suspension, the dried dye was redissolved in a minute quantity of diluent.

(iii) *Actual test on lymphocytes.* For testing, two drops of the lymphocyte suspension (see above) were added to a 5 × 0·8 cm siliconed tube. These tubes were centrifuged, the supernatant removed and antiglobulin reagent added to the deposited lymphocytes. The contents of the tubes were mixed and incubated at 4° for 1 hour with periodic agitation.

After incubation a little diluent was added to each tube before gentle centrifugation (200 ***g*** for 5 minutes) and washing the deposited lymphocytes three times in 0·75 ml diluent. After the third wash the lymphocytes were resuspended in one drop which was transferred to a fresh 5 × 0·5 cm siliconed tube.

One drop of the appropriate indicator cell suspension (0·4 per cent) was added, the cells mixed and then centrifuged at 300 ***g*** for 2 minutes. This brought the cells down into a compact button at the bottom of the tube. After discarding half the supernate the deposited cells were resuspended by gentle pipetting action and transferred to a glass slide and placed on the small drop of dissolved toluidine blue dye. This was again mixed by gentle pipetting action and then a siliconed coverslip was placed on top and the edges sealed with wax.

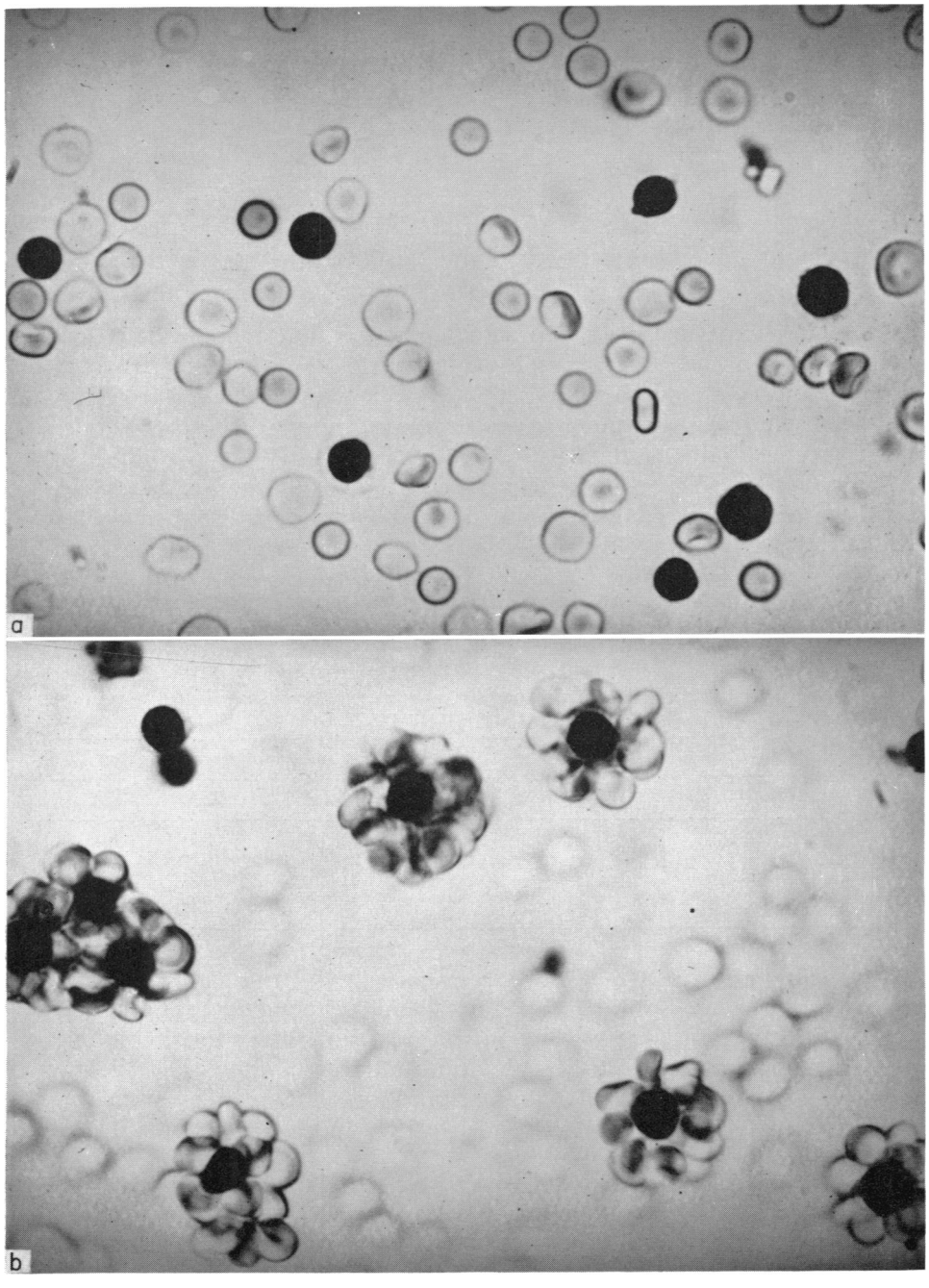

FIG. 2. Appearance under the microscope (bright field × 10 eyepiece and × 25 objective) on reading the mixed antiglobulin reaction. Lymphocytes are stained with toluidine blue, red cells are unstained. (a) Negative reaction, (b) positive reaction with approximately 70 per cent reacting lymphocytes, i.e. being rosetted by immunoglobulin-carrying red cells.

After allowing the cells to settle and the lymphocytes to take up the dye, counts were made of the rosetted and unrosetted lymphocytes (see Fig. 2). The occasional polymorph which got through in the preparation could be easily recognized and disregarded. The lymphocytes were clearly recognizable after staining with toluidine blue and could be seen easily as the centre of the red cell rosettes or as individually free cells.

RESULTS

DEMONSTRATION OF IgG DETERMINANTS AND ESTIMATION OF THE NUMBER OF REACTING LYMPHOCYTES

In preliminary experiments it was soon apparent that there was a decided dose-dependence between the concentration of the antiglobulin reagent used to treat the lymphocyte suspension and the number of lymphocytes forming rosettes. In the first experiment reported here, lymphocyte preparations from two different rabbits were each exposed to varying dilutions: 1 : 10, 1 : 30, 1 : 90 and 1 : 270 of two different anti-IgG sera, C17 produced in a sheep and A58 produced in a goat. After incubation and washing the treated lymphocytes were brought into contact with indicator red cells sensitized with IgG antibody. The numbers of IgG-reacting lymphocytes (expressed as per cent) resulting from treatment with the different doses of antibody are given in Table 1. With both reagents there was a fairly constant number with dilutions up to 1 : 90 with a sharp fall off

TABLE 1

IgG DETERMINANTS ON RABBIT LYMPHOCYTES: DOSE DEPENDENCE SHOWN IN TITRATION OF ANTI-IgG REAGENTS

Lymphocytes	Anti-rabbit IgG reagent	IgG-reacting lymphocytes (per cent)			
		1 : 10*	1 : 30	1 : 90	1 : 270
Rabbit 23/58	Sheep C17	67	66	62	8
	Goat A58	52	47	47	2
Rabbit 20/72	Sheep C17	24	25	36	8
	Goat A58	32	26	25	2

* Dilution of anti-IgG reagent.

with more dilute reagent. Rabbit 23/58 showed a higher percentage of reacting cells than did rabbit 20/72. Addition of rabbit IgG at concentrations down to at least 1 μg/ml to the treated lymphocytes before addition of the indicator red cells abolished all rosette formation. Likewise no rosettes formed if unsensitized red cells were used in the indicator system. With three normal sheep sera and two normal goat sera, instead of sheep anti-rabbit IgG, no rosette-formation occurred.

Next, lymphocytes were prepared from fourteen normal rabbits and the number of IgG-reacting lymphocytes measured using a saturating dose, namely a 1 : 30 dilution of the sheep C17 anti-IgG reagent. The results, as percentages of IgG-reacting lymphocytes in the individual rabbits, were 63, 54, 42, 40, 35, 33, 30, 29, 27, 26, 24, 15, 13 and 6, respectively. Thus there was considerable variation in the number of IgG-reacting lymphocytes—the range varying from 63 to 6 per cent. Whether a small or large percentage of cells reacted, the individual rosettes were similar in appearance.

DEMONSTRATION OF γ- AND μ-SPECIFIC DETERMINANTS AND DETERMINANTS RECOGNIZED BY ANTI-Fab

The anti-γ, anti-μ and anti-Fab reagents were prepared as described under Methods. The general procedure for testing was as already described with the following differences. When using the anti-γ reagent, the indicator red cells were sensitized with IgG sheep cell antibody in a DEAE exclusion fraction. When using the anti-μ reagent the indicator red cells were sensitized with IgM sheep cell antibody in a Sephadex G-200 exclusion fraction. Again, bearing in mind a likely dose-dependence, the lymphocytes from two rabbits were tested using a 1 : 10, 1 : 20, 1 : 40 and 1 : 80 dilution of the two reagents. The number of lymphocytes forming rosettes in the four titrations is given in Table 2. With both

TABLE 2

IMMUNOGLOBULIN DETERMINANTS ON RABBIT LYMPHOCYTES REVEALED WITH ANTI-γ, ANTI-μ AND ANTI-Fab REAGENTS: DOSE-DEPENDENCE SHOWN IN TITRATION REAGENTS

Lymphocyte	Specific reagent	Indicator red cells carrying	Reacting lymphocytes (per cent)			
			1 : 10*	1 : 20*	1 : 40*	1 : 80*
R25/70	Anti-γ	IgG	38	33	24	29
	Anti-μ	IgM	28	18	4	2
	Anti-Fab	Fab	52	54	49	35
R58/45	Anti-γ	IgG	43	50	14	11
	Anti-μ	IgM	15	21	20	5
	Anti-Fab	Fab	49	40	30	8

*Dilution of specific reagent.

reagents adequate saturation was obtained using a 1 : 20 dilution of the reagent; in most cases there was a falling off in numbers of reacting cells when the reagent was used more dilute. The specificity of the two systems was shown by switching over the indicator cells; no rosettes were formed when lymphocytes exposed to anti-γ were subsequently brought into contact with red cells sensitized with IgM antibody, nor *vice versa* when anti-μ-treated lymphocytes were brought into contact with IgG-coated red cells (Table 3).

The same two lymphocyte suspensions were also tested against an anti-Fab reagent at dilutions 1 : 10 to 1 : 80. The indicator red cells were sensitized with Fab fragments of rabbit anti-sheep red cell antibody prepared by pepsin and mercaptoethanol treatment. The numbers of reacting lymphocytes are also shown in Table 2. A 1 : 20 dilution of the reagent appeared to saturate fully all the sites on the lymphocytes. In this system addition of Fab in a concentration of 2 μg/ml to the treated lymphocytes before adding the indicator cells completely inhibited the reaction.

Lymphocytes from ten rabbits were then tested for immunoglobulin determinants detectable with these reagents specific for γ, μ and Fab. To saturate the number of sites (see Table 2) all the reagents were used at a 1 : 20 dilution. The results are given in Table 3. The anti-γ and anti-μ systems showed absolute specificity on most lymphocytes tested although with three lymphocyte preparations some slight reaction was observed with the heterologous indicator cell. In eight out of the ten lymphocyte suspensions the number of Fab-reacting cells was greater than either the γ- or μ-reacting cells but less than the total of γ- plus μ-reacting cells. With one lymphocyte suspension (Rabbit 79/75) the reaction with anti-Fab was excessively low; this peculiar result was repeated with the same finding. In some rabbits μ-reacting lymphocytes predominated while in other rabbits there were

TABLE 3

ENUMERATION OF LYMPHOCYTES WITH IMMUNOGLOBULIN DETERMINANTS REACTING WITH ANTI-γ, ANTI-μ AND ANTI-Fab EACH IN APPROXIMATELY SATURATING DOSE

Lymphocytes	Reacting lymphocytes (per cent)						
	Anti-γ*		Anti-μ*		Anti-Fab*		
	IgG†	IgM†	IgG	IgM	Fab	IgG	IgM
R23/54	15	0	0	38	40	40	
R23/56	20	2	0	36	40	47	
R23/58	23	0	0	36	59		40
R79/75	24	0	1	10	2	2	
R82/08	29	0	0	14	27		20
R19/49	30	0	0	21	30	12	
R25/70	35	0	0	18	54		
R79/68	41	0	0	13	75		10
R20/92	47	3	0	25	57		50
R58/45	50	NT	NT	21	40		

0, <1; NT, not tested.

* Reagent.

† Indicator cell carrying.

more γ-reacting cells. There was some tendency for an inverse relationship between the two types of reacting cells. Our experiments at this stage have no direct bearing on the question as to whether both μ and γ determinants exist on the one cell.

ATTEMPTS TO ENUMERATE LYMPHOCYTES REACTING WITH ANTI-Fab AND ANTI-Fc DETERMINANTS WITH THE USE OF A POLYVALENT SERUM

It should be possible to enumerate the number of μ- γ- and Fab-reacting lymphocytes by incubating the lymphocytes with a polyvalent antiserum (sheep 207 anti-whole rabbit serum) and controlling specificity by choice of sensitized indicator red cells (e.g. SRBC carrying IgG or IgM antibody) and specific inhibitors such as IgG or Fab in the last stage of the test.

A number of experiments were performed in this way and the results of one are given in Table 4. Repeated tests showed great variation and this testing procedure was discarded because the measurement of γ-sites demanded a different serum concentration from that optimal to measure μ-sites; also the combining antibodies of different specificity could well interfere sterically with each other.

EXAMINATION OF CIRCULATING AND LYMPH-NODE LYMPHOCYTES AND THYMOCYTES FROM THREE RABBITS

These were preliminary tests to see if IgG- or μ-specific determinants could be shown under the existing conditions of testing on rabbit thymocytes and to see if lymph node cells differed markedly from circulating lymphocytes.

Three rabbits were bled in the usual manner for preparation of circulating lymphocytes. The rabbits were then killed and the thymuses and mesenteric lymph nodes removed. These were cleaned up and cut finely between two sharp scalpel blades to free their cells into Tris–Hanks's solution containing 0·2 per cent BPA. After allowing clumps of cells to settle the cells in fine suspension were centrifuged, washed twice in diluent and stored in glycerol–Hanks's solution in the Linde container over liquid nitrogen till tested.

TABLE 4

ATTEMPT TO ENUMERATE LYMPHOCYTES REACTING WITH ANTI-γ, ANTI-μ AND ANTI-Fab USING A POLYVALENT ANTIGLOBULIN REAGENT

Polyvalent antiglobulin reagent	Indicator red cells	Inhibitor	Determinant being measured	Reacting lymphocytes (per cent)	
				R25/70	R58/45
1 : 10	IgG	None	IgG+Fab	64	26
		Fab*	γ	18	3
		IgG†	–	0	0
	IgM	None	IgM+Fab	25	36
		Fab	μ	43	44
		IgG	μ	32	37
	Fab	None	Fab	44	40
		Fab	–	0	0
		IgG	–	0	0
1 : 20	IgG	None	IgG+Fab	34	12
		Fab	γ	22	16
		IgG	–	0	0
	IgM	None	IgM+Fab	39	60
		Fab	μ	5	28
		IgG	μ	19	45
	Fab	None	Fab	22	20
		Fab	–	0	0
		IgG	–	0	0

* Pepsin $Fab'_2 \simeq 40$ μg/ml.
† IgG (Mann) 200 μg/ml.

The circulating lymphocytes, lymph node lymphocytes and thymocytes were tested for IgG- and μ-specific determinants on the one day. The number of reacting cells is given in Table 5. The anti-IgG testing system would detect γFc and Fab determinants of all immunoglobulin classes while the anti-μ testing system was specific for Fc determinants on IgM. Practically no IgG or μ reacting cells were detected in the thymus suspensions. In rabbit C31 about 1 per cent of the thymus cells gave an IgG-reaction and with the others also a few reacting cells were observed but their number was not greater than 0·5 per cent. In all cases there were fewer reacting cells in the mesenteric lymph node than in the circulation. The ages of the three rabbits, C31, 51/33 and 25/70 were $\frac{1}{2}$, $1\frac{1}{2}$ and 2 years, respectively.

TABLE 5

IgG AND μ-SPECIFIC DETERMINANTS ON CIRCULATING LYMPHOCYTES, LYMPH NODE CELLS AND CELLS FROM THE THYMUS

Cells under test from rabbit	Anti-globulin reagent	Indicator red cells	Reacting lymphocytes or thymocytes (per cent)		
			Circulating lymphocytes	Lymph node cells	Cells from thymus
C31	Anti-IgG	IgG	26	16	1
25/70	Anti-IgG	IgG	61	25	0
51/33	Anti-IgG	IgG	47	13	0
25/70	Anti-μ	IgM	14	4	0
51/33	Anti-μ	IgM	28	2	0

0 = < 1. The mesenteric node was the source of lymph node cells.

As4 AND As6 LIGHT CHAIN ALLOTYPIC DETERMINANTS ON THE CIRCULATING LYMPHOCYTES OF INDIVIDUAL RABBITS

After preliminary experiments which showed the presence of these determinants on the lymphocytes of rabbits of the appropriate gentoypes the anti-As4 and anti-As6 reagents (made in b5b6 and b4b5 rabbits, respectively) were titrated to find the dose-dependence between the number of reacting cells recorded and concentration of reagent used. The results of such standardizations on the lymphocytes of three rabbits are shown in Table 6. Two indicator red cell suspensions were prepared, one with sheep red cells

TABLE 6

ALLOTYPIC DETERMINANTS As4 AND As6: DOSE-DEPENDENCE BETWEEN NUMBER OF REACTING LYMPHOCYTES AND CONSIDERATION OF ANTIBODY REAGENT

Lymphocyte and genotype	Antibody reagent	Indicator red cell carrying	Reacting lymphocytes (per cent)			
			1 : 10*	1 : 20*	1 : 40*	1 : 80*
R83/21 (a2a3b4b6)	Anti-As4	As4	37	35	26	16
R83/21 (a2a3b4b6)	Anti-As4	As6	0			
R83/18 (a3a3b4b6)	Anti-As4	As4	11	11	8	3
R83/18 (a3a3b4b6)	Anti-As4	As6	0			
R83/21	Anti-As6	As4	0			
R83/21	Anti-As6	As6	13	9	10	7
R83/18	Anti-As6	As4	0			
R83/18	Anti-As6	As6	14	13	10	0
R83/21	Anti-L	As4	26	27	6	
R83/21	Anti-L	As6	12			
R83/18	Anti-L	As4	17			
R83/18	Anti-L	As6	5			
R83/21	Anti-Fab	As4	34	35	25	26
R83/21	Anti-Fab	As6	18	12	9	3
R83/18	Anti-Fab	As4	36	34	27	18
R83/18	Anti-Fab	As6	10	8	3	0

*Dilution of antibody reagent.

sensitized with a 1 : 10,000 dilution of a rabbit As4 anti-sheep cell serum and one with red cells sensitized with a 1 : 10,000 dilution of a rabbit As6 anti-sheep cell serum. Rosette-formation occurred with anti-As4-treated lymphocytes only with indicator cells carrying the As4 determinant and *vice versa* with anti-As6-treated lymphocytes rosette-formation occurred only with indicator cells carrying the As6 determinant. This established the specificity of the test system. A plateau in dose-dependence relationship was found and in further tests both reagents were used in a 1 : 20 dilution.

In previous experiments by Sell and Gell (1965b) on blast transformation of *in vitro* cultured lymphocytes by anti-allotype sera the evidence was that the determinants were actually synthesized by the lymphocytes and were not passively adsorbed *in vivo* on to the lymphocytes from the plasma. In the present experiments most of the lymphocytes are 'killed' during their separation from the blood and preparation for testing. This is done purposely with the object of haemolysing the red cells during the glycerol treatment so that these ghosts may be removed from the lymphocyte preparation. It was conceivable that immunoglobulin could be absorbed passively from the serum on to damaged lymphocytes during the preparative procedure. The following experiment in which lymphocytes from one rabbit were bathed in the serum of another showed this not to be the case.

Two rabbits R82/92 and R79/75 homozygous b4b4 and b6b6, respectively, were bled and the blood defibrinated. Each blood was divided into two lots and the cells (red and white) deposited by centrifugation. The supernatant serum (6 ml) was discarded and one sample of each resuspended in 6 ml R82/92 serum (homozygous b4b4) and the other in 6 ml R79/75 serum (homozygous b6b6). These serum samples were obtained by bleeding the same rabbits the previous day. The cells were resuspended and allowed to stand for 1 hour at room temperature and a further hour at 37°. There were now four samples—As4 lymphocytes in As4 serum, As4 lymphocytes in As6 serum, As6 lymphocytes in As4 serum and As6 lymphocytes in As6 serum. The lymphocytes then went through the usual preparative procedure and were stored at −100° overnight in 20 per cent glycerol saline and then recovered the next day for testing. There was no evidence for the passive uptake on to the lymphocytes of the immunoglobulin allotype of the serum of the opposite rabbit. This suggested that the immunoglobulin determinants being measured on the lymphocytes as tested, were integral to the lymphocytes themselves: though further tests are in progress to confirm this.

When testing the dose-dependence of the anti-As4 and anti-As6 allotype sera, titrations were also set up with the anti-Fab and anti-Light chain sera as it was expected that in the latter cases similar counts of reacting lymphocytes would be obtained, irrespective of whether the indicator red cells were sensitized with either As4 or As6 sheep cell antibody. However, as may be seen from Table 6 this was not the case, for in each instance the counts with As4 indicator cells were much higher than those with As6 indicator cells. The IgG source from which the light chains and Fab were prepared originated from pooled rabbit serum and both the anti-light chain serum and anti-Fab worked equally well in antiglobulin tests on red cells sensitized with either As4 or As6 carrying antibody. Despite this, the evidence suggests that these reagents possessed a predominating proportion of antibody to the As4 determinant.

TABLE 7

EXPERIMENT SHOWING APPARENT PREDOMINANT ROLE OF ANTI-As4 ALLOTYPIC SYSTEM IN TESTS ON LYMPHOCYTES WITH HETEROLOGOUS GOAT ANTI-RABBIT Fab REAGENT

Indicator red cells carrying	Dilution of red cell antibody in indicator system	Reacting lymphocytes (per cent)	
		1 : 10*	1 : 20*
As4	1 : 5,000	38	43
	1 : 10,000	39	50
	1 : 20,000	51	43
As5	1 : 5,000	23	21
	1 : 10,000	23	27
	1 : 20,000	27	25
As6	1 : 2,500	22	17
	1 : 5,000	22	22
	1 : 10,000	33	24
	1 : 20,000	26	19

* Lymphocytes R83/18 (a3a3b4b6) treated with goat anti-Fab.

That the effect could not be explained by the As6-carrying indicator red cells being more weakly sensitized than the As4 indicator cells, is shown by the experiment recorded in Table 7. In this experiment lymphocytes from an a3a3b4b6 rabbit were treated with a 1 : 10 and 1 : 20 dilution of sheep anti-Fab and the washed lymphocytes tested against

red cells treated with varying dilutions of rabbit antibody carrying either allotypic determinant As4, As5 or As6. The higher counts were found only with the As4 carrying indicator cells and this was irrespective of the strength of the red cell sensitization.

The lymphocytes of a series of rabbits were then examined to get an estimate of the number of As4-reacting and As6-reacting lymphocytes in the individual bloods. As in the preliminary standardization experiments each lymphocyte suspension was also tested with the anti-Fab reagent and in all cases two indicator red cell suspensions were used, one carrying As4 sheep cell antibody and the other As6 sheep cell antibody (a 1 : 10,000 dilution of each being used for sensitization).

TABLE 8

ALLOTYPIC DETERMINANTS As4 AND As6: NUMBER OF REACTING LYMPHOCYTES, IN PER CENT, IN THIRTEEN NORMAL RABBITS

Rabbit lymphocytes and genotypes	Reacting lymphocytes (per cent)					
	Anti-As4*		Anti-As6*		Anti-Fab*	
	As4†	As6†	As4	As6	As4	As6
R82/57 (a3a3b6b6)	0	0	0	27	8	3
R82/65 (a2a3b6b6)	0	0	7	35	12	2
R82/59 (a2a2b6b6)	0	0	6	25	15	8
R79/75 (a3a3b6b6)	0	0	4	34	23	13
R83/18 (a3a3b4b6)	11	0	0	16	34	8
R82/08 (a1a2b4b5)	33	0	0	0	38	21
R83/21 (a2a3b4b6)	35	0	0	9	35	12
R23/58 (a1a3b4b6)	41	0	2	12	58	24
R19/49 (a2a3b4b4)	47	0	0	0	57	28
R58/45 (a3a3b4b6)	51	0	7	30	62	46
R23/56 (a1a2b4b6)	52	0	3	37	61	32
R23/54 (a2a3b4b6)	56	0	0	24	70	44
R51/33 (not grouped)	59	0	0	0	65	39
R20/72 (a1a3b4b4)	66	0	0	0	72	47
R25/70 (not grouped)	69	0	0	0	78	44
R79/68 (a2a2b4b4)	75	0	0	0	68	31

* Reagent.
† Indicator red cell carrying.

The results are gathered together in Table 8. The As4 testing system appeared quite specific as between As4 and As6 and in As4-positive animals the number of As4-reacting lymphocytes varied between 11 and 75 per cent. In this series there were four As4-negative rabbits. In the As6 system, reacting cells varied between 12 and 37 per cent. However, despite the fact that no reacting cells were found in As6-negative rabbits the anti-As6 reagent was also reacting with another system as shown by occasional cross-reactions with indicator red cells lacking As6. Subsequent experiments with another anti-As6 reagent (made in b5b5 animals) eliminated these aberrant reactions. As noted previously, a higher percentage of lymphocytes reactive with anti-Fab (usually double) was found with the As4 carrying indicator cells than with As6 carrying cells.

DISCUSSION

The results given in this paper are mainly concerned with technique and standardization.

Although the *prima facie* conclusion from these results is that the Ig determinants detected on non-metabolizing or dead cells by the technique used are products of those cells,

and identical with the determinants which are targets and triggers for blast transformation, it would be premature to speculate about their role until this point is more thoroughly investigated. Should such be the case however, this system would be ideal for the investigation of the simultaneous manifestations on cells of different Ig class determinants, and of the presence or absence of 'allelic exclusion' on normal lymphocytes. Neither these nor the other experiments quoted throw any direct light on the question whether such determinants are identical with the 'receptors' of antigen-sensitive or primed cells.

Clearly many other experiments need to be done on this system, both to increase the specificity and range of the antisera, and to investigate the situation in species other than the rabbit. These experiments are under way.

It is nevertheless gratifying to recall earlier experience with the mixed antiglobulin reactions when it was being used to measure leucocyte iso-antibodies in man. Chalmers, Coombs, Gurner and Dausset concluded a paper in 1959 by stating: 'Finally it should be added that, up to the present, we have not been able to adapt the method to the study of iso-antigens on rabbit leucocytes, which behave as if globulin is either firmly absorbed on the surface, or forms an integral part of the cell membrane.'

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REFERENCES

CHALMERS, D. G., COOMBS, R. R. A., GURNER, B. W. and DAUSSET, J. (1959). 'The mixed antiglobulin reaction in the detection of human iso-antibodies against leucocytes, platelets and HeLa cells.' *Brit. J. Haemat.*, **5**, 225.

CHAPLIN, H., COHEN, F. and PRESS, E. M. (1965). 'Preparation and properties of the peptide chains of normal human 19S γ-globulin (IgM). *Biochem. J.*, **95**, 256.

COOMBS, R. R. A. and GELL, P. G. H. (Eds.) (1968). *Clinical Aspects of Immunology*, Chapter I. Blackwell Scientific Publications, Oxford.

DUBISKI, S., DUDZIAK, Z. and SKALBA, D. (1959). 'Serum groups in rabbits.' *Immunology*, **2**, 84.

PERNIS, B., CHIAPPINO, G., KELUS, A. S. and GELL, P. G. H. (1965). 'Cellular localization of immunoglobulins with different allotype specificities in rabbit lymphoid tissues.' *J. exp. Med.*, **122**, 853.

SELL, S. (1967a). 'Studies on rabbit lymphocytes in vitro. V. Induction of blast transformation with antisera to rabbit IgG subunits.' *J. exp. Med.*, **125**, 289.

SELL, S. (1967b). 'Studies on rabbit lymphocytes in vitro. VI. Induction of blast transformation with sheep antisera to rabbit IgA and IgM.' *J. exp. Med.*, **125**, 393.

SELL, S. and GELL, P. G. H. (1965a). 'Studies on rabbit lymphocytes in vitro. I. Stimulation of blast transformation with anti-allotype antiserum.' *J. exp. Med.*, **122**, 423.

SELL, S. and GELL, P. G. H. (1965b). 'Studies on rabbit lymphocytes in vitro. IV. Blast transformation of lymphocytes of new born rabbits.' *J. exp. Med.*, **122**, 923.