STUDIES ON RABBIT LYMPHOCYTES IN VITRO

II. Induction of Blast Transformation with Antisera to Six IgG Allotypes and Summation with Mixtures of Antisera to Different Allotypes*

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Antisera directed against the As4 allotype of rabbit gamma globulin are capable of inducing blast transformation and DNA synthesis in lymphocyte cultures obtained from the peripheral blood of As4 rabbits (1). The present study describes analogous experiments using antisera against the other five well characterised allotypic determinants: As1, 2, 3, 5, and 6 (2). In addition, the effect of mixtures of antisera to two different allotypic determinants was compared with the effect of mixtures of two antisera to the same allotype. The results demonstrate that antisera to all six allotypes are effective in the induction of blast transformation and that mixtures of antisera to two different allotypes result in a summation of response over and above mixtures of two antisera to the same allotype.

Materials and Methods

In general the same methods were used as in the previous paper (1).

Antisera.—The anti-As4 antisera were from the same animals as those used previously, but after a further course of immunization. The other antisera were raised by the method of Dubiski *et al.* (3), in collaboration with Dr. A. S. Kelus. One antiserum (see Table I) was raised in an As1, 4 rabbit with antibody gamma globulin from an As3, 5 rabbit, and had strong antibody against both As3 and As5. Other antisera, against the As3 determinant, though satisfactory for typing, tended to be weak and of poor avidity. Where relevant, the allotype of the donor of the antiallotype serum is recorded in the Tables. In cases where antisera were mixed, as in the summation experiments (Tables IV, V, and VI), it was clearly essential that they should not interreact; *i.e.*, neither antiserum could be raised in a rabbit having an allotypic specificity to which the other antiserum might be directed.

It is known that antisera against As5 and As6 tend to cross-react to a small extent when raised in As4 animals. The consequence of this cross-reaction is referred to in the Results section below.

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Haemagglutination Titres of Antisera.—These were carried out using plastic dishes with sheep red cells sensitised with subagglutinating doses of antisera raised in animals of the appropriate allotype. One drop of a 2.5 per cent red cell suspension was added to one drop of diluted antiserum plus three drops of saline. The titres were recorded as the final serum dilution that gave agglutination.

TABLE I

Relationship of Lymphocyte Transformation by Antiallotype Serum to Allotypes of Rabbit IgG

Serum	Allotype of donor cells							
	1, 3, 4, 5	1, 3, 5	2, 4	2, 3, 4, 6	3, 4	1, 6	1, 3, 6	2, 3, 6
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Blast transformation								
Autologous....	<1	<1	<1	<1	<1	<1	<1	<1
Anti-1.....	14	17	—	<1	<1	16	3	<1
Anti-2.....	<1	—	9	5	—	—	—	2
Anti-3.....	<1	<1	—	<1	<1	—	<1	<1
Anti-3+5.....	—	—	—	14	9	—	2	13
Anti-4.....	26	<1	47	28	29	—	—	—
Anti-5 (+6)...	8	27	<1	3	—	2	—	7
Anti-6.....	<1	—	—	9	<1	21	10	20
C^{14}-thymidine uptake (disintegrations/10 minutes)								
Autologous....	608	613	404	182	344	283	618	478
Anti-1.....	3090	4930	—	294	435	2990	7320	680
Anti-2.....	308	—	573	633	—	—	—	885
Anti-3.....	457	347	—	200	578	—	1775	515
Anti-3+5.....	—	—	—	2983	1390	—	2540	7320
Anti-4.....	7360	344	19,950	3021	1708	—	—	—
Anti-5 (+6)...	1260	12,070	138	452	—	1287	—	2980
Anti-6.....	246	—	—	868	309	8400	8070	7100

RESULTS

Transformation with Antiallotype Sera.—The percentage blast transformation and the C^{14}-thymidine uptake of lymphocyte cultures derived from eight animals of different allotype, when exposed to the range of available antiallotype antisera, are recorded in Table I. All the cell preparations responded, as judged by transformation or thymidine uptake or both, with the exception of the anti-As3 serum, which was known to be a weak one. To check whether it might be impossible to stimulate through the As3 determinant, an antiserum was used which reacted strongly with As3, but also had anti-As5 specific activity. Tested on four As3 positive/As5 negative animals, this antiserum produced a significant response by both the criteria used. The failure of the more specific anti-As3 serum to stimulate must therefore have been due to its low potency. Other antisera, as described elsewhere, have been found to lose their effect on

moderate dilution (1). An antiserum specific for a macroglobulin allotype (see reference 4) was also tested; this produced no blast transformation when added to cultures of the appropriate lymphocytes. However, since this antiserum was relatively weak, the negative result may have little significance.

TABLE II

Agglutination of Donor Cells with Antiallotype Sera

Sera	Allotype of donor cells			
	1, 3, 5	1, 6	2, 4	3, 4
Anti-1	0	0	0	0
Anti-2	±	0	0	0
Anti-3	+	+	0	+
Anti-4	0	0	0	±
Anti-5	+	+	+	++
Anti-6	0	0	+	+
Autologous	+	0	+	+
Autologous + PHA	+++	+++	+++	+++

TABLE III

Effect of Homozygosity and Heterozygosity of Donor Cells on Degree of Lymphoblast Formation

Donor anti-A4 serum	Allotype of donor cells	Blast formation	C^{14}-thymidine uptake*	Haemagglutination titre of serum
		per cent		
5626	1, 3, 4	31	5,300	$<1/128 \cdot 10^3$
	1, 3, 4, 5	26	5,140	
5627	1, 3, 4	27	13,180	$1/128 \cdot 10^3$
	1, 3, 4, 5	32	4,240	
5590	1, 3, 4	18	2,230	$>1/64 \cdot 10^3$
	1, 3, 4, 5	15	2,800	

* Disintegrations per 10 minutes.

A few cultures from which a positive result might be expected were marginally or not at all significant by one test or the other, but never by both (for example the As2, 3, 6 and the As2, 4 animals with anti-As2). On the other hand the anti-As5 serum, raised in an As4 homozygous animal, showed weak serological cross-reactivity with As6; this was reflected in the induction of transformation of lymphocytes obtained from As6 positive/As5 negative donors, though the homologous reaction was always stronger. It should be noted

that there was at least a twofold variation in the responses of the various cell preparations to an identical antiserum.

Failure of Antiallotype Antisera to Cause Cytagglutination.—In the previous paper we described experiments (1) designed to exclude the possibility that cells react to antiallotype sera by virtue of a passive coating of gamma globulin. It was demonstrated that anti-As4 serum had no agglutinating activity when tested against As4 cells. Table II records an experiment in which most of the

TABLE IV

Summation of Lymphoblast Transformation with Antisera to As1 and As6

Milliliters of antisera per culture*		Degree of transformation			
		Allotype of donor cells			
Anti-As1 in As3, 5‡	Anti-As6 in As3, 5‡	As1, 6		As1, 2, 4, 6	
		Blast formation	C^{14}-thymidine uptake§	Blast formation	C^{14}-thymidine uptake§
		per cent		*per cent*	
—	—	<1	441	<1	727
0.5	—	41	13,700	11	5,560
—	0.5	28	12,850	7	5,175
0.25	—	22	12,110	14	9,960
—	0.25	14	13,500	8	3,660
0.1	—	19	15,760	13	13,120
—	0.1	12	4,190	6	4,840
0.5	0.5	63	33,860	23	17,690
0.25	0.25	56	21,370	12	11,700
0.1	0.1	53	46,650	27	28,300
0.25	0.1	57	58,350	20	18,750
0.1	0.25	51	58,100	34	23,400

* Total serum volume in all cultures made up to a minimum of 0.5 ml with normal As3, 5 serum.

‡ Allotype of antiserum donor.

§ Disintegrations per 10 minutes.

antisera used in the present experiments were tested against cells containing between them all six allotypes. Such agglutination as occurred was minimal, and was quite unrelated to the allotypic specificity of the cells.

Stimulation with Anti-As4 Serum of Cells Homozygous or Heterozygous for As4.—One might anticipate that more cells should respond to a given antiserum in a homozygous than in a heterozygous animal. Examination of the data in Table III, which was designed to illuminate this problem, and a comparison with the data in Table IV shows the difficulty of coming to any definite

conclusion on this point. Although a glance at Table IV, (primarily designed to illustrate "summation", as discussed below) would suggest that the homozygote responds strikingly better, with respect to allotypic determinants both at the a and at the b locus, the data given in Table III using three different anti-

TABLE V

Summation of Lymphoblast Transformation with Antisera to As5 and As6

Milliliters of antisera per culture*		Degree of transformation			
		Allotype of donor cells			
		As2, 3, 5, 6		As1, 5, 6	
Anti-As5 in As1, 3, ‡	Anti-As6 in As3, 4‡	Blast formation	C^{14}-thymidine uptake§	Blast formation	C^{14}-thymidine uptake§
		per cent		*per cent*	
—	—	<1	316	<1	202
1.0	—	43	8,140	17	9,750
—	1.0	17	6,290	27	7,950
0.5	—	34	7,450	28	11,600
—	0.5	42	4,120	32	7,000
0.25	—	43	3,140	34	11,400
—	0.25	44	3,770	46	6,250
0.1	—	37	5,760	25	4,610
—	0.1	42	6,109	10	2,229
0.5	0.5	69	11,200	43	21,600
0.25	0.25	65	6,250	53	17,400
0.1	0.1	58	20,270	49	11,400
0.5	0.25	67	13,550	43	12,830
0.25	0.5	53	10,400	55	8,570
0.25	0.1	59	12,230	54	7,050
0.1	0.25	57	6,350	43	9,160

Note. Neither of the antisera used in this experiment produced cross-reactive transformation of lymphocytes of the other allotype.

* Total serum volume in all cultures 1.0 ml. Volume made up with normal As1, 4 serum when necessary.

‡ Allotype of antiserum donor.

§ Disintegrations per 10 minutes.

sera against As4 shows no real difference between the responses of As1, 3, 4 and As1, 3, 4, 5, cells. The "stimulability" of cell preparations is, as quoted above, so variable that it would be necessary to compare large numbers of homozygous and heterozygous cell preparations to arrive at a statistically significant conclusion.

Summation of Stimulation by Antisera to Two Different Determinants.—Light

may be thrown upon the question as to whether cells can respond by virtue of all or any of the determinants represented in their genotype, or by virtue of only one of them, with a different type of experiment, in which the effects of two antisera of differing specificity are allowed to "summate". In Tables IV and V are recorded the results of two experiments, the first with mixtures of antisera to allotypes from different loci, the second with mixtures to allotypes from the same locus. In all cases there was clear evidence that the effects of the combined stimuli exceeded the effects of stimulation by comparable total

TABLE VI

Absence of Summation with Mixtures of Two Antisera Against the Same Allotype (As4)

Milliliters of Antisera Added per Culture*		Degree of transformation of As4 donor cells	
5626 (1/5) (in As1, 3, 5, 6)‡	5590 (in As1,3, 5, 6)‡	Blast formation	C^{14}-thymidine uptake§
		per cent	
—	—	<1	380
0.5	—	54	4620
—	0.5	46	2995
0.25	—	34	2370
—	0.25	35	3670
0.1	—	3	520
—	0.1	24	1100
0.5	0.5	43	2310
0.25	0.25	35	2040
0.1	0.1	31	1990
0.25	0.1	26	1870
0.1	0.25	31	2230

* The total serum volume of all cultures made up to a minimum of 0.5 ml with normal rabbit serum of As1, 3, 5, 6.

‡ Allotype of antiserum donor.

§ Disintegrations per 10 minutes.

amounts of antiserum against one determinant alone. Thus, the combination of two 0.1 ml doses was always more effective than a single 0.25 ml dose, of two 0.25 ml doses than a single 0.5 ml dose, and so on. In contrast, combination of two different antisera against the same (As4) determinant was no more effective than would be expected from the total volume of antiserum present (Table VI).

DISCUSSION

The basic mechanism by which antiallotype sera stimulate lymphoblast formation *in vitro* is unknown. The available evidence from attempts to coat cells passively with heteroallotypic gamma globulin (1), from agglutination tests (see reference 1 and Table II above) from tests with fluorescent antibody (5),

and from the finding that incubation of the appropriate antiserum and cells for 45 minutes is necessary to induce the full degree of transformation (1) suggests that the stimulus is not merely an antigen-antibody reaction occurring on the cell surface, though this cannot be said to be fully established.

It is a reasonable assumption that a proportion of circulating lymphocytes can produce IgG (6); thus it is not irrational that a specific anti-gamma globulin should produce the transformation results as described above. Nor is it irrational that cells should be most ready to produce one type only of the types of IgG potentially represented in the genotype, or even a single polypeptide chain of IgG. In myeloma, for example, some cells produce only light chains and form Bence-Jones proteins of one antigenic type (7).

The specificity of the formation of allotypic gamma globulins by rabbit plasma cells is still not clear. Colberg and Dray (8) working with animals heterozygous at one locus (As4, 5) tentatively concluded from fluorescent antibody studies that individual cells all produced both As4 and As5 simultaneously. Recently, however, Pernis, Chiappino, Kelus, and Gell (9) analysing antibody production both in As4, 5 and As4, 6 animals have found, that in the cells of the lymph node pulp at least, about equal numbers of cells were making each determinant but never making both. If a given plasma cell in a heterozygote only produces gamma globulin specific for one determinant, it must be assumed that only one chromosome of a pair is activated to protein synthesis; it may be a random matter which chromosome is activated. Can we therefore analyse the data on summation (Tables IV to VI) in terms of the hypothesis that cells are predetermined or "primed", even at the normal lymphocyte stage, to respond (in a still undefined way) in terms of one particular chromosome of the pair? If this were so one would find two populations of cells, each with its characteristic dose-response curve to the appropriate antiallotypic serum. Maximal stimulation by an antiserum to one determinant would then always be less alone than when combined with maximal, or submaximal, stimulation by an antiserum to another determinant. The data in Table V are consistent with this assumption, though in many cases the stimulation is still greater than would be expected.

It is very much more puzzling that "summation" appears to occur between allotypes from *different* loci, (Table IV), especially in the case of the doubly homozygous animal (As1, 6). Here again the cells behave as if they belonged to two distinct populations primed to make either As1 or As6; yet every cell capable of making the whole gamma globulin molecule is presumably able to synthesise both allotypic determinants. However, summation of allotypic blast transformation using antisera to allotypes controlled by different loci might result from a failure of the expression of one of the chromosomal loci in a given cell. Pernis *et al.* (9) have observed that, of the plasma cells in a homozygote that stained with fluorescent anti-IgG, up to 30 per cent did not stain with fluorescent antiallotype sera. From similar findings with IgG by Bornstein

and Oudin (10), *i.e.* that not all IgG of a given homozygote contain allotypic determinants, such cells containing IgG, but not the allotypic determinant, have been termed a- or b- depending on the locus controlling the missing allotypic determinant. Therefore, if a similar mechanism is operative in regard to lymphocytes, the lymphocytes of the As1, 6 rabbit may be distributed among the following possible categories; a+b+, a+b−, a−b+, and a−b−. Approximately 30 per cent of the plasma cells examined by Pernis *et al.* (9) were a−, and 15 per cent b−. If such data can be extrapolated to peripheral lymphocytes then there should usually be a greater amount of transformation with anti-b allotype sera than with anti-a allotype sera. The data presented in Table IV are not consistent with this, but in general we have found that the amount of transformation produced by antisera to b locus determinants greater than that by antisera to a locus determinants; although this is also a function of the strength of the individual antisera.

Because the amount of blast transformation induced by antiallotype sera as measured by per cent transformation is not really quantitative, any attempts to assess distribution of lymphocytes according to the above categories may be subject to considerable error. However, from the maximum transformation data found for the lymphocyte cultures from the As1, 6 rabbit given in Table IV, the following lymphocyte distribution may be constructed: As a maximum of 63 per cent transformation occurs with mixtures of anti-a and anti-b sera, 37 per cent of the lymphocytes must be non-reactive, or a−b−. From the percentage transformation using individual antisera, a maximum of 41 per cent of the lymphocytes must be a+, and a maximum of 28 per cent b+. Therefore:—

$$(a+b+) + (a+b-) = 41 \text{ per cent}$$

$$(a+b+) + (a-b+) = 28 \text{ per cent}$$

and from the results with antisera mixtures:

$$(a+b+) + (a+b-) + (a-b+) = 63 \text{ per cent}$$

If these three equations are solved for the three unknowns, it can be calculated that almost all of the transformation observed may be accounted for by a+b− and a−b+ type lymphocytes. Thus, there may be no or very few a+b+ cells at the lymphocyte level. If the a locus controls determinants located on the H chain of IgG, and the b locus controls determinants on the L chain (see Feinstein, Gell, and Kelus, 11) these calculations suggest that lymphocytes may be capable of making only one or the other of the two polypeptide chains of IgG.

SUMMARY

Specific antisera directed against all six of the well characterised allotypic determinants of rabbit IgG (As1, 2, 3, 4, 5, and 6) are capable of inducing blast

transformation and DNA synthesis when added to lymphocyte cultures obtained from donor rabbits having the appropriate IgG allotype. Mixtures of antisera directed against two different allotypic determinants induce a "summation" of transformation and DNA synthesis over and above the effect of mixtures of two antisera directed against the same allotypic determinant. This summation effect is observed regardless of whether the antisera which have been mixed are directed against allotypic determinants controlled by the same locus or by different loci. The finding that summation occurs with mixtures of two antisera directed against both the allotypic determinants of a double homozygote rabbit (As1, 6) suggests that lymphocytes from the peripheral blood may be primed to produce only one or the other of the two polypeptide chains of IgG, but not both.

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