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Lymphocyte-Transforming Activity of Homologous and Heterologous Antisera to Rabbit Leucocytes

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Summary. Two out of nine rabbits receiving a course of immunization with homologous leucocytes produced antisera which caused blast formation and stimulated DNA synthesis of donor lymphocytes *in vitro*. Of the seven rabbits which failed to produce active antisera three had received only a short immunization course. The active antisera 'transformed' lymphocytes from some rabbits but not those of others. This stimulatory effect was not related to the red cell types or the γ-globulin allotypes of the rabbits.

Heterologous antisera raised to rabbit leucocytes in rats and guinea-pigs were cytotoxic to rabbit leucocytes *in vitro* and also caused leucoagglutination but never stimulated DNA synthesis and blast formation.

INTRODUCTION

Rabbit peripheral lymphocytes transform *in vitro* into blast cells when stimulated with phytohaemagglutinin or staphylococcal filtrate in a manner comparable with that of human lymphocytes (Knight, Ling, Sell and Oxnard, 1965). Gräsbeck, Nordman and de la Chapelle (1965) found that sera from rabbits immunized with human leucocytes caused blast formation and initiated DNA synthesis when added to human leucocytes in culture. Holt, Ling and Stanworth (1966) found that the relative blastogenic activity of the heterologous leucocyte antisera was not dependent upon the source of the cultured human leucocytes. On immunological grounds it might be expected that specific lymphocyte antisera capable of distinguishing lymphocytes containing certain antigens would be more likely to be obtained by homologous rather than heterologous immunizations. Homologous leucocyte antisera have been prepared in rabbits, and have indeed been found to be blastogenic towards the lymphocytes of some rabbits but not of others. It is possible that antisera prepared and tested in this way may be useful for 'typing' lymphocytes on the basis of specific blastogenesis.

MATERIALS AND METHODS

Animals

Stock laboratory rabbits, guinea-pigs and rats were used; the rabbits were from various sources and included New Zealand Whites, New Zealand Reds and Dutch Crosses.

Lymphocyte suspensions

Rabbits were bled from the marginal ear vein into sterile siliconed bottles and the blood was gently defibrinated. It was then mixed with a half-volume of 3 per cent gelatin (fine

grain Pig-skin, British Glue and Chemicals Ltd) and left for 1–2 hours at 37° to sediment the erythrocytes, a technique based on that of Coulson and Chalmers (1963). The leucocyte-rich plasma was then removed, the cells spun down and washed in saline and either resuspended in 0·5 ml saline for injection or in sterile rabbit serum and Eagle's medium for cell cultures. Using this method over 90 per cent of the leucocytes were lymphocytes but there was some contamination with erythrocytes (about 5 erythrocytes/leucocyte).

Immunization schedules

(a) *Homologous immunizations.* Eighteen rabbits were chosen at random and nine were used as leucocyte donors and the others as recipients. The first injection consisted of $50–60 \times 10^6$ live leucocytes in saline injected into the marginal ear-vein. Subsequent injections consisted of $20–30 \times 10^6$ leucocytes. A course of five weekly injections was given initially in each case and six of the recipients, after 2 or 6 weeks, received a further course of weekly immunizations.

(b) *Heterologous immunizations.* Six rats each received two weekly injections of $20–30 \times 10^6$ live rabbit leucocytes in saline in the footpads. Two of these received two further weekly injections and two received four further injections.

Two guinea-pigs received two weekly intraperitoneal injections of $20–30 \times 10^6$ live rabbit leucocytes and two others received six injections.

One rabbit was bled by heart puncture after injection of nembutal and the blood lymphocytes separated in the usual way. The mesenteric lymph nodes, the bone marrow from the femur, the thymus, spleen and liver were removed. Cell suspensions prepared from the lymph nodes, bone marrow, thymus and spleen were stored in Eagle's medium at 4° before use. The microsomal fraction of the liver cells, which was separated by differential centrifugation of a homogenate prepared in 10 per cent sucrose, was the fraction sedimenting between 10,000 and 100,000 ***g***. Twelve guinea-pigs received three intraperitoneal injections, two animals with each of the cell suspensions and two with liver microsomes in Freund's complete adjuvant (Difco).

Antisera

Before the injection course and a week after each injection recipient rabbits were bled from the marginal ear vein and the serum obtained was stored at −20° before testing. Rats and guinea-pigs were bled by heart puncture after two injections and at the end of the immunization course and the sera obtained were again stored at −20°. All the antisera were then tested for their effect on rabbit lymphocytes *in vitro*.

(a) *Cell cultures.* Cultures were set up in bijoux bottles each containing 5×10^6 leucocytes from donor or unrelated rabbits. The culture medium consisted of 15 per cent normal rabbit serum (either fresh or complement-inactivated by incubation at 56° for 30 minutes) in Eagle's minimum essential medium (containing 10 per cent tryptose broth and penicillin and streptomycin). Recipient serum before the injection course or after leucocyte injection was added to concentrations varying between 1/1000 and 1/10. The total culture volume in each case was 3 ml. The cultures were harvested after 3 days of incubation, 0·12 μc of [^{14}C]thymidine (thymidine, $^{14}C_2$, 15–30 mc/mM, Amersham) being added 24 hours before harvesting in some cases. Some cultures were spun down and smears made for morphological examination. Others were spun and the cells washed and prepared for scintillation counting. The cells were washed twice in saline, twice in 5 per cent trichloroacetic acid and twice in methanol. The deposit was finally suspended in 1 ml

methanol in each case and this suspension added to 10 ml of a phosphor prepared by adding 1 volume of Triton X100 (Lennig Chemical Co. Ltd, London, W.C.1) to 2 volumes of xylene containing POPOP (0·012 per cent) and PPO (0·6 per cent), a technique based on that of Patterson and Green (1965). The preparations were counted for 10 minutes each in a Packard Tricarb scintillation counter and the counts corrected for colour and chemical quenching using the channel-ratio method.

(b) *Tests for leucocytotoxic antibody, haemagglutinins and leucoagglutinins.* Viability of cells was assessed by the dye-exclusion test using a method similar to that of Black and Berenbaum (1964) using Eosin Y. The following procedure was found to give good reproducible results. Two standard drops of cell suspension (containing 5×10^6 cells/ml) were incubated in the presence of 2 drops of fresh autologous serum and 2 drops of the antiserum for 1 hour at 37° in an atmosphere of 5 per cent CO_2 in air. One drop of Eosin Y in saline (8 mg/ml) was then added and, after a further incubation for 15 minutes in the presence of the dye, counts of the viable unstained cells and dye-penetrated cells were made.

The leucoagglutination test of Dausset as described by Van Rood (1962) was used. Two drops of leucocyte suspension were incubated for 2 hours at 37° with 2 drops of the antiserum. The supernatant was then removed and 1 drop of 6 per cent acetic acid was added to the sediment to lyse the red cells. The leucoagglutination was then assessed microscopically.

Erythroagglutination titres were determined by doubling dilution of the antisera in 0·1 ml volumes of 10 per cent normal inactivated rabbit serum in isotonic saline in MRC pattern Perspex agglutination trays. A 2 per cent suspension of washed rabbit erythrocytes (0·1 ml) was added and the agglutination titre read by the 'settled pattern' technique after an interval of 2–3 hours.

Typing of erythrocytes

In early experiments the technique of Joysey (1959) was employed using antisera kindly provided by Dr Joysey. In later experiments whole blood samples preserved in acid citrate–dextrose were sent to Dr Carl Cohen of Cleveland, Ohio for typing (Cohen and Tissot, 1965).

γ-Globulin allotypes

These were kindly determined by Professor P. G. H. Gell using a gel precipitation technique with specific antisera as described by Dray, Dubiski, Kelus, Lennox and Oudin (1962).

RESULTS

HOMOLOGOUS IMMUNIZATIONS

Of the serum samples taken from the nine recipient rabbits after a course of five immunizations only one showed definite activity in cultures of donor lymphocytes as shown by the formation of blast cells and increased uptake of [^{14}C]thymidine (Table 1, Donor/Recipient pair 4). Activity was detectable at an antiserum concentration of 1/100 and 1/10 in cultures containing inactivated serum. With complement present no increased [^{14}C]thymidine incorporation was observed and no blast cells were seen. About 10 per cent of the mononuclear cells in complement-free cultures were morphologically blast cells although some of these had cytoplasmic blebs and others appeared to have been mechanically damaged by the process of smearing (Figs. 2 and 3).

After seven injections two other recipient rabbits had produced antisera which activated peripheral donor lymphocytes in cultures free from complement. Donor/Recipient combination 7 (Table 1) gave a marginal but reproducible increase in [^{14}C]thymidine incorporation after seven injections of leucocytes but unfortunately further immunizations could not be carried out as the donor animal died. Combination 9 showed a more marked increase.

TABLE 1

DNA SYNTHESIS OF DONOR LYMPHOCYTES INCUBATED WITH RECIPIENT SERUM

Pair	Donor	Recipient	Counts/minute with serum after injection							
			0	3	4	5	6	7	8	10
1	NZW	NZW	132	127	74	133				
2	DC	NZW	50	98	34	88				
3	NZW	NZW	131	105	60	50				
4	DC	NZW	45	50		532	116	15,200	24,000	
5	DC	NZW	157	81		81	120	60	129	119
6	DC	NZW	50	111		14	57	43	0	59
7	NZW	NZR	126	100		99	43	240*		
8	NZW	NZR	44	126		38	49	13	67	91
9	DC	NZR	50	70		46	182	800	38	3000

Each figure represents the mean counts per minute in triplicate cultures of donor cells in the presence of recipient serum before and after injection of leucocytes in each of the nine combinations. The antiserum dilution in the cultures was 1/100. NZW, New Zealand White; NZR, New Zealand Red; DC, Dutch Cross.

* Donor animal died at this point.

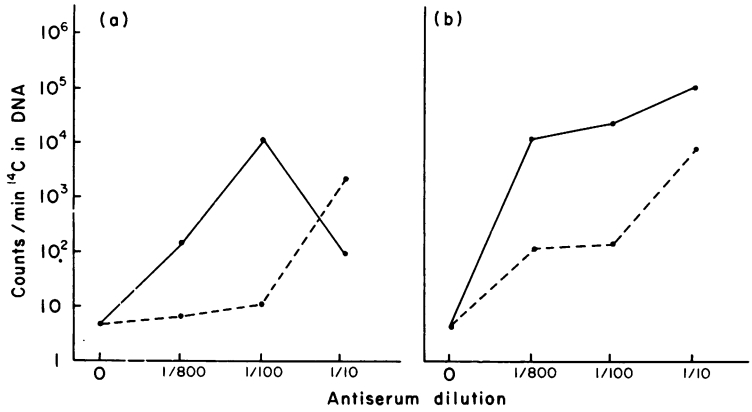

FIG. 1. The effect of recipient serum 4 on the DNA synthesis of donor cells before (- - - -) and after (——) the eighth booster injection. (a) Fresh serum, (b) inactivated serum. DNA synthesis in donor lymphocytes is indicated by scintillation counts/minute. The effect of different concentrations of fresh and inactivated serum on donor lymphocytes before and after the eighth injection is shown.

Fig. 1 shows the effect of recipient serum 4 on the transformation of donor cells before and after the eighth booster injection. When the serum in the cultures was inactivated there was an increased [^{14}C]thymidine incorporation into donor cells in the presence of the antiserum after seven and eight injections over a wide range of antiserum concentrations. However, when complement was present no response to antiserum was seen in the donor lymphocytes *in vitro* until after the seventh injection although this was not so marked as the

response seen in inactivated serum. After the eighth injection there was still a stimulatory effect when active antiserum was present but this antiserum became toxic at concentrations above 1/100. In cultures where inactivated serum 4 after the eighth injection was present at a concentration of 1/10 approximately 30 per cent of the cells were morphologically 'transformed'.

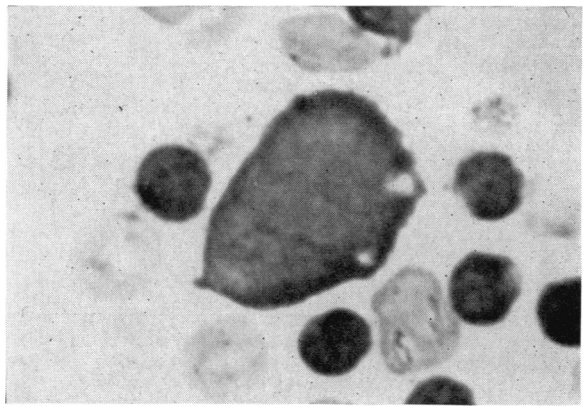

FIG. 2. Typical 'transformed' donor rabbit lymphocyte in the presence of antiserum 4.

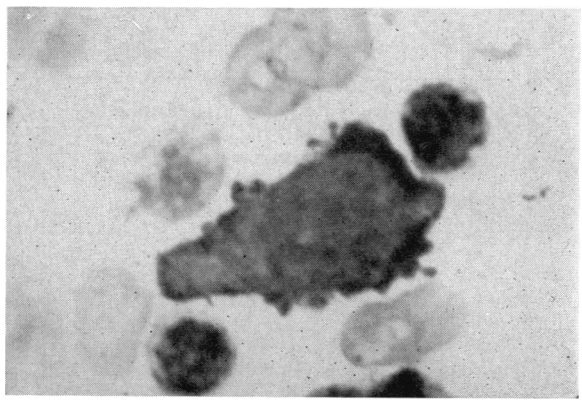

FIG. 3. Distorted lymphocyte in culture of donor lymphocytes incubated with antiserum 4.

It was not surprising to find that erythroagglutinins were also present in some of the antisera as there was considerable erythrocyte contamination in the injected leucocyte suspensions. It was striking, however, that only the two sera which gave a good lymphocyte transforming effect caused agglutination of donor red cells. This raised the possibility that an antibody to a red cell antigen might be involved (Table 2). Using the dye-exclusion test for cytotoxic antibody these two sera were also the only ones which showed any cytotoxic effect after a 1¼-hour incubation period with antiserum (Table 2).

As will be seen from Table 3, the transforming activity of antiserum 4 did not appear to be due to its isohaemagglutinin (which was consistent with Cohen's anti-A) or to any antibody to a γ-globulin allotype. Scintillation counts of 7360 and 14,800 were observed

with lymphocytes from two of the eight animals incubated in the presence of a 1/10 concentration of antiserum 4. The lymphocytes from three other animals showed slight but reproducible increase of DNA synthesis (from 2 to 5 times control values) and the

TABLE 2

THE EFFECTS OF RECIPIENT SERA ON DONOR CELLS AFTER THE INJECTION COURSES

Donor/Recipient pair	Counts/minute	Erythroagglutination titre	Cytotoxicity (per cent dead)
1	133	< 4	1
2	88	< 4	0
3	50	< 4	0
4	24,000	8	8
5	119	< 4	1
6	59	< 4	0
7	240	< 4	1
8	91	< 4	0
9	3000	24	6
Control values	30–157	< 4	0–3

The counts per minute are the mean counts from triplicate cultures of donor lymphoctyes incubated in 1/100 concentration of antiserum. All serum in the cultures was inactivated. Erythroagglutination titres are those of the antisera with donor rabbit red cells. The cytotoxic effect of the antisera on donor leucocytes is represented by the percentage dead as judged by dye-exclusion. The range covered by all the control values is given in each case.

TABLE 3

THE EFFECT OF ANTISERUM 4 ON THE LYMPHOCYTES AND ERYTHROCYTES FROM A RANDOMLY SELECTED PANEL OF RABBITS

Blood group (Cohen)	Allotype	Counts/minute	Erythroagglutination titres	Cytotoxicity (per cent dead)
ACFLM*	As 345	100,000	8	8
ACFLM	As 14	140	4	0
CFLM	As 134	14,800	< 4	4
CFLM	As 234	205	< 4	4
CFM	As 14	438	< 4	5
CFM	As 134	190	< 4	1
ACFHM	As 134	550	32	1
ABCFLM	As 345	7360	8	19
ACFM	As 134	87	32	1
Control values		54–200	< 4	1–3

The red cell types and allotypes of the rabbits are shown in the first two columns. The [^{14}C]thymidine incorporation into lymphocytes from these animals in the presence of a 1/100 concentration of antiserum 4 is shown in column 3. The erythroagglutinin titres with serum 4 using red cells from these rabbits are in column 4 and the cytotoxic effect of antiserum 4 on leucocytes from the rabbits as judged by dye exclusion is shown in the last column. The control values were obtained using serum from recipient rabbit 4 before the injection course and the three tests were performed on cells from each rabbit.

* Donor rabbit.

three others were negative. There was a partial correlation of activity with cytotoxic effect of the antibody although the percentage of dead cells after a 1¼-hour incubation period was not very high in any instance. Leucoagglutination tests were not found to be accurately reproducible in our hands but there appeared to be some degree of leucoagglutination with most of the antisera.

HETEROLOGOUS IMMUNIZATIONS

No blast formation or increased [^{14}C]thymidine incorporation into rabbit peripheral lymphocytes was observed with either fresh or inactivated serum from any of the immunized animals over a very wide range of concentrations. Table 4 exemplifies the results with the fourteen guinea-pigs immunized; no significantly raised scintillation counts were observed from preparations of rabbit lymphocytes which had been cultured in the presence of the antiserum. The results with rat anti-rabbit sera were similar.

TABLE 4

DNA SYNTHESIS IN CULTURES OF RABBIT LYMPHOCYTES CONTAINING INACTIVATED SERA FROM IMMUNIZED GUINEA-PIGS

Immunizing cells	Counts/minute	
Spleen	75	60
Thymus	80	46
Bone marrow	98	168
Lymph node	89	156
Peripheral	129	124
Peripheral	144	38
Liver microsomes	109	209
None	100	166

Each figure represents the mean counts per minute of duplicate cultures containing a concentration of 1/100 of antiserum from a single animal.

TABLE 5

EFFECT OF RAT AND GUINEA-PIG ANTISERA ON RABBIT CELLS

Antiserum present	Counts/minute	Erythroagglutination titre	Cytotoxicity (per cent dead)
None	33–95	< 4	1–4
Rat anti-rabbit			
1	25–105	32–128	10–19
2	45–139	32–512	7–26
Guinea-pig anti-rabbit			
1	35–105	4–128	5–17
2	66–140	4–64	5–18

Each test was performed in triplicate with each serum on cells from five to eight randomly selected rabbits.

The sera from the two rats and two guinea-pigs which had received courses of six injections of rabbit peripheral cells were investigated for other properties (Table 5). All the sera gave erythroagglutination titres with donor red cells and with the red cells of all rabbits tested. Very marked leucoagglutination was also observed with all four sera against all rabbits tested and in the presence of complement the sera were toxic to leucocytes. Cells from five to eight animals were tested with each serum and the range of response found is shown in Table 5. Absorbing out the erythroagglutinating antibody by incubation with rabbit erythrocytes before culturing rabbit lymphocytes with serum made no difference to the level of incorporation of [^{14}C]thymidine.

DISCUSSION

The experiments using homologous antisera to rabbit lymphocytes show that certain of these will distinguish between lymphocyte types within the species by causing transformation of the lymphocytes of some rabbits but not those of others. The length of the leucocyte injection course may be an important factor in determining the strength and activity of these antisera and it seems possible that with prolonged injection courses further lymphocyte-stimulating antisera may have been produced, particularly in the three animals which had received fewer injections. Because of this, no conclusions can be drawn as to the frequency of factors responsible for producing these 'active' antisera or the possible range of their effect. The nature of the homologous leucocyte antibody responsible for the activation has not been studied but Holt *et al.* (1966) have shown that in heterologous antisera to human leucocytes possessing lymphocyte activating properties the activity is in the IgG fraction. The activating antibody to allotypic rabbit γ-globulin reported by Sell and Gell (1965) was also shown to be in the IgG fraction.

It is premature to attempt to extrapolate these findings in rabbit to the human situation. However, in the human field several methods of detection of the leucocyte groups have been used and it now appears that the seven groups identified by Teresaki, Mickey, Vredevoe and Goyette (1965), using a cytotoxicity test are similar to those identified by other workers (Amos, 1965; Dausset, Ivanyi and Ivanyi, 1965; Bodmer and Payne, 1965; Schulman, Marder, Hiller and Collier, 1964; Van Rood, Van Leeuwen, Schippers, Vooys, Frederiks, Balner and Eernisse, 1965). Teresaki, Vredevoe, Porter, Mickey, Marchiord, Faris, Heremann and Starzl (1966) have concluded that these major leucocyte groups in man correspond to histocompatibility antigens and are 'intermediate strength antigens' which may be involved to varying degrees in histocompatibility. This suggestion has been based on studies on the post-graft sera of patients receiving kidney homografts. It would be of great interest to examine leucocyte antisera, either from patients receiving multiple transfusions or tissue grafts, or from women after multiple pregnancies, for a 'transforming' effect on lymphocytes; preferably on lymphocytes from the cell or tissue donor.

Sell and Gell (1965) showed that specific antisera to rabbit γ-globulin from hyperimmunized rabbits would cause transformation of lymphocytes from rabbits of the donor allotype. Although immunization with homologous leucocytes under the conditions described here is unlikely to produce antibody to allotypic γ-globulins, tests were performed to confirm that these were not present. Both the donor and recipient of pair 9 (Table 1) were in fact of the same allotype (As 134) and the antiserum of this combination had a good transforming effect on the donor lymphocytes. As seen in Table 3, the transforming effect of antiserum 4 also showed no correlation with the allotypes involved. In many cultures, too, autologous donor serum was present which might be expected to effectively 'neutralize' any antibody to serum factors. There was no precipitin line visible on Ouchterlony analysis of the antiserum and donor serum.

The stimulatory effects of the rabbit leucocyte antisera *in vitro*, sometimes even when complement was present, is in contrast to leucopenic effects observed *in vivo* (Nagaya and Sicker, 1966; Woodruff and Anderson, 1964). In the latter instance the interference with the immune apparatus of the host is greater than can be accounted for by simple lymphocytolysis.

Mixtures of homologous human leucocytes from genetically dissimilar human beings give rise to the transformation of a proportion of the lymphocytes to blast cells capable

of DNA synthesis (Bach and Hirschhorn, 1964; Bain, Vas and Lowenstein, 1964; Rubin and Stenzel, 1964; Aisenberg, 1965; Moynihan, Jackson and Hardy, 1965; Oppenheim, Whang and Frei, 1965). Harris, Clarke, Jones, Sheppard, Lehane, McCarthy, Lawler and Shatwell (1966) have not, however, found a convincing correlation between the mixed cell response and the normal lymphocyte transfer test although they do not exclude this possibility.

Marshall, Rigo and Melman (1966) have stressed the importance of producing a one-way effect where cells from one source only are stimulated and have partially solved this problem by mixing macrophages from one person with the lymphocytes of another. The lymphocyte typing of rabbit cells which is indicated by our experiments is somewhat more simple to interpret than a mixed cell test where cells from both sources can transform to varying extents, as it represents a completely one-way effect; lymphocytes from only one source are present in the cultures. It is probable that if a large number of specific antisera were prepared by the immunization procedures described the technique would provide a new source of information about lymphocyte and possible transplantation antigens. In 1946 Medawar showed that the intradermal injection of leucocytes into rabbits induced homograft sensitivity and Friedman, Retan, Marshall, Henry and Merrill (1961) and Rapaport, Lawrence and Converse (1964) have reported similar findings in man. There is an increasing body of evidence to suggest that leucocyte isoantigens are closely related to transplantation antigens and they are, therefore, of practical importance in the problem of histocompatibility testing. Engelfriet, Heersche, Eijsvoogel and van Loghem (1966) using a cytotoxic antibody test have concluded that it is likely that only antigens common to granulocytes and lymphocytes can be important transplantation antigens. Kapitchnikov, Ballantywor and Stetson (1962), from experiments on skin transplantation in rabbit have pointed out that in rabbits, as in humans, the major histocompatibility antigens are present on the lymphocyte but not on the erythrocyte.

It is clear from the experiments with homologous antisera described here and the experiments with heterologous lymphocyte antisera described by Holt *et al.* (1966) that only some leucocyte antigens occupy 'trigger sites'. It is interesting that an investigation of the activation of sea-urchin eggs by rabbit antibody (Perlmann, 1959) showed clearly that only one out of four egg antigens identified was a 'trigger' antigen for parthenogenetic division. The activation of lymphocytes by antisera also has points of resemblance with the enhancement of the growth of certain tumours by antibodies (Kaliss, 1962).

Möller and Möller (1962) have shown that cells sensitive to the cytotoxic action of antisera contain a high concentration of antigenic receptors on the cell surface. Möller and Eklund (1965) have detected red cell antigens on lymphoid cells using a fluorescence technique and have studied the cytotoxic effect of antibody to these antigens. The combined action of antibodies directed against ABO and Rh antigens caused the death of the target lymphoid cells whereas A, B or Rh antisera used singly did not have this cytotoxic effect. This seems to indicate a situation similar to the mouse system of the H2 type where an increase of the concentration of the reactive sites by combining several antisera causes formerly insensitive cells to become sensitive to the cytotoxic test. Oda and Puck (1961) have illustrated that cell killing is a function of the concentration of the complement as well as of antibody. Thus, the 'trigger' sites for activation would add their contribution to that of the other sites on the donor lymphocytes to give a high density of antibody-occupied sites with consequent lysis in the presence of complement. In several homologous rabbit antisera where no transforming effect was observed leucoagglutinins were present

but on the addition of complement these were not toxic. Presumably, in these cases, the density of reactive sites was not great enough to produce cytotoxicity. Low concentrations of active antisera in the presence of complement were not toxic but produced good stimulation in some instances and this, too, is in agreement with the suggestion that density of antigenically combined sites is an important factor in the cytotoxic effect. Although the heterologous antisera contained strong leucoagglutinins and haemagglutinins they failed to activate rabbit lymphocytes *in vitro*. This is possibly due to a high concentration of non-activating antibodies which, even in the absence of complement, were toxic in the dilutions usually used in the cultures and so possibly may have masked any activating effect of the antibody to the 'trigger' sites.

Gräsbeck *et al.* (1964) found that one of the rabbit anti-human leucocyte sera was not mitogenic until some months after the end of the immunization course and this he suggested was due to the toxic effect of the high leucoagglutinin titre.

All the anti-leucocyte sera so far reported as having a positive transforming effect on leucocytes were prepared by intravenous leucocyte injections. In the heterologous systems tested here the injections were intraperitoneal or into the footpads and the significance, if any, of this difference is being investigated.

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