

Transformation of Rabbit Lymphocytes by Anti-Allotype Serum: Ultrastructure of Transformed Cells and Suppression of Responding Cells by Foetal Exposure to Anti-Allotype Serum

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Summary. The transformation of rabbit lymphocytes by anti-allotype serum (AAS) has been confirmed. The transformed cells closely resembled at the ultrastructural level those obtained by phytohaemagglutinin stimulation. The cytoplasm contained abundant free ribosomes with virtually no rough surfaced endoplasmic reticulum. A feature peculiar to AAS stimulated cultures was the presence of an amorphous material in and around the blast cells which might represent immune complexes of AAS with its antigen. The suppression of immunoglobulin allotype in animals heterozygous at a given locus by foetal exposure to AAS has been confirmed. It was further shown that this allotypic suppression was accompanied by gross deficiency in the number of peripheral blood lymphocytes which transformed in the presence of the AAS to which the animal had initially been exposed *in utero*.

INTRODUCTION

Genetically controlled antigenic differences occur on rabbit immunoglobulin molecules (Oudin, 1960; Dray, Dubiski, Kelus, Lennox and Oudin, 1962). These antigens, or allotypes, are controlled by two known loci which segregate independently (Dray *et al.*, 1962; Kelus and Gell, 1967). There are at least three alleles (Aa1, Aa2 and Aa3) for the *a* locus and four alleles (Ab4, Ab5, Ab6 and Ab9) for the *b* locus (Dray *et al.*, 1962; Dubiski and Muller, 1967; Kelus and Gell, 1967).

Individual immunoglobulin molecules from homozygous or heterozygous animals express only one allotypic specificity for each locus (Dray and Nisonoff, 1963; Dray, Young and Nisonoff, 1963). About 85–90 per cent of the serum immunoglobulins have the Ab4 (or Ab5) specificity when an animal is homozygous at the *b* locus for one of these allotypes; in a heterozygous Ab4,5 animal the ratio of Ab4 to Ab5 molecules is approximately 2 : 1 (Dray and Nisonoff, 1963; Bornstein and Oudin, 1964; Stemke, 1965; Kelus and Gell, 1967).

Cells also carry allotypic markers. Thus peripheral blood lymphocytes in culture undergo transformation into 'blast' cells and mitosis in response to specific stimulation by anti-allotype sera (AAS) prepared in other rabbits (Sell and Gell, 1965; Gell and Sell, 1965; Sell, Rowe and Gell, 1965). Plasma cells may be shown to produce immunoglobulins

with given allotypic specificities by immunofluorescent staining with AAS (Pernis, Chiappino, Kelus and Gell, 1965; Cebra, Colberg and Dray, 1966). Individual plasma cells express only one allotypic marker for each locus and the relative number of cells possessing a given specificity roughly corresponds with the serum immunoglobulin concentration of that allotype (Pernis *et al.*, 1965; Cebra *et al.*, 1966).

The relative and absolute levels of immunoglobulin molecules bearing the two allotypic specificities in the serum of an animal heterozygous at a given locus can be dramatically altered by foetal or neonatal exposure to an AAS directed against one of the allotypes (Dray, 1962; Mage and Dray, 1965). Thus progeny of a homozygous Ab5,5 buck and a homozygous Ab4,4 doe with high titre antibodies to allotype Ab5, have a greatly, and sometimes completely, reduced level of Ab5 immunoglobulins and a compensatory increase in Ab4 immunoglobulins. The concentration of Ab5 serum immunoglobulins gradually increases with time but a complete return to normal levels has not so far been demonstrated (Mage and Dray, 1965). Similarly, the number of plasma cells producing immunoglobulin molecules of the two specificities may be altered in a manner and degree comparable to the serum immunoglobulin levels by exposure to the appropriate AAS in early life (Lumms, Cebra and Mage, 1967).

The mechanism of this 'allotypic suppression' is not understood, nor is it known whether cells other than those directly involved in the production of serum immunoglobulins are affected by exposure to AAS. The following study was undertaken to determine whether the numbers of peripheral blood lymphocytes expressing allelic specificities, as demonstrated by 'anti-allotype transformation', were also altered in allotypically suppressed animals. Furthermore, the ultrastructure of lymphocytes transformed by AAS was examined and compared with that of lymphocytes transformed by another mitogenic agent—phytohaemagglutinin.

MATERIALS AND METHODS

Preparation of antisera

Anti-allotype sera were prepared by immunizing rabbits of one allotype with antibody of another allotype coated onto killed *Proteus vulgaris* organisms as described by Dubiski, Dudziak, Skalba and Dubiska (1959). In this study an Ab5,6 rabbit was immunized with Ab4 antibody for the anti-Ab4 serum and an Ab4,4 rabbit was immunized with Ab5 antibody to raise an anti-Ab5 serum. Goat antiserum to rabbit IgG was kindly supplied by Dr G. Torrigiani; this was monospecific as judged by immunoelectrophoresis.

Measurement of immunoglobulin concentrations

The concentrations of serum IgG and serum immunoglobulins of allotypes Ab4 and Ab5 were determined using single radial diffusion in agar (Mancini, Carbonara and Heremans, 1965). Briefly, antisera were incorporated in agar to give a final dilution of 1 : 40. Serial dilutions up to 1 : 16 of the sera to be tested were placed in wells in the agar, and after incubation overnight the precipitin ring diameters were measured. A standard curve was prepared for each plate by including dilutions of purified preparations of rabbit serum IgG obtained by anion-exchange chromatography of rabbit sera of allotypes Ab4,4, Ab5,5 and Ab4,5 on DEAE-cellulose in 0.02 M phosphate buffer, pH 6.8. Immunoelectrophoresis in agar was used to demonstrate the purity of the preparations. The protein

concentrations were determined using the relationship $E (0.5 \text{ cm}/280 \text{ m}\mu 0.1) \equiv 126 \mu\text{g}$ IgG/ml (McDuffie and Kabat, 1956) and adjusted to 2.0 mg/ml. In calculating the concentration of serum allotypes in the rabbits tested it was assumed that 88 per cent of the molecules of the standard IgG preparations were positive for the *b* locus specificity (Dray and Nisonoff, 1963; Bornstein and Oudin, 1964; Stemke, 1965; Kelus and Gell, 1967). Thus, the standard Ab4 IgG which contained 2.0 mg IgG/ml was taken to contain 1.7 mg Ab4 IgG/ml.

Lymphocyte cultures

Twenty millilitres of blood were withdrawn from the rabbit medial ear artery and added to two heparinized universal container bottles. The erythrocytes were sedimented with 0.5 volumes of 3.5 per cent dextran T250 (Pharmacia Ltd) in saline for 30 minutes at 37° and the lymphocyte-rich upper layer collected and spun in plastic tubes at 150 *g* for 10 minutes. The cell button was washed twice with Eagle's minimum essential medium (EMEM) and resuspended in 10 ml EMEM. Cell counts were made in 2 per cent acetic acid. Cell concentrations were adjusted to give a final suspension of 1×10^6 lymphocytes/ml in EMEM with 20 per cent inactivated rabbit serum of a non-reactive allotype. One-millilitre cultures were set up in triplicate in Bijou bottles with phytohaemagglutinin (PHA) or in 'half-dram' screw-cap vials (diameter 1 cm; Anchor Glass Ltd) with AAS. These containers were found to provide conditions for optimal transformation with the respective mitogens. The surface area and the volume of the vial were both one-third those of the Bijou bottle. These two factors altered both the cell density and the oxygen tension in the cultures (Ling, 1968). Sterile containers, equipment and solutions were used throughout. All sera were sterilized by passage through Millipore membranes.

Optimal doses of AAS were found to be 0.035 ml/culture of the anti-Ab4 serum and 0.10 ml/culture of the anti-Ab5 serum. Anti-Ab4 serum was added to cultures containing normal serum of allotype Ab5,6 and anti-Ab5 serum was added to cultures with Ab4,4 normal serum in order to avoid cross-reaction of the AAS with the normal serum (Kelus and Gell, 1967). Controls with both normal sera were included routinely. Normal sera varied somewhat in their ability to support growth and transformation of the cells and an effort was made to use the same rabbits as normal serum donors throughout the experiments. The optimal dose of PHA (Burroughs Wellcome) was 0.015 ml/culture of the reconstituted preparation.

The cultures were gassed with 5 per cent CO₂/air and incubated at 37° for 48 hours at which time 0.125 μC [¹⁴C]thymidine (specific activity 55.8 mc/mm; Radiochemical Centre, Amersham) in 0.025 ml saline were added to each container. The cultures were incubated a further 16 hours before harvesting.

Lymphocyte stimulation was assessed by determining both the percentage of lymphocytes transformed into blast cells and the uptake of radioactive thymidine. Cell smears were prepared by taking 0.5-ml aliquots from one culture in each triplicate; the cells were washed twice, resuspended in phosphate-buffered saline and spread on glass slides by spinning at 450 rev/min for 15 minutes in a centrifugal apparatus (Doré and Balfour, 1965). The cell preparations were fixed for 10 minutes with methanol and stained with May-Grünwald-Giemsa. At least 200 cells were counted on each slide to determine the percentage of transformed lymphocytes. Cultures were prepared for liquid scintillation counting to measure [¹⁴C]thymidine uptake by the method of Ling and Holt (1967). Only cultures with more than 50 per cent of cells surviving were harvested.

Electron microscopy

Lymphocyte cultures were prepared and incubated as described above. The cells were washed three times with EMEM and fixed with cacodylate buffered 3 per cent glutaraldehyde followed by osmium tetroxide and embedded in Araldite. Sections were examined in an AEI-EM6B electron microscope.

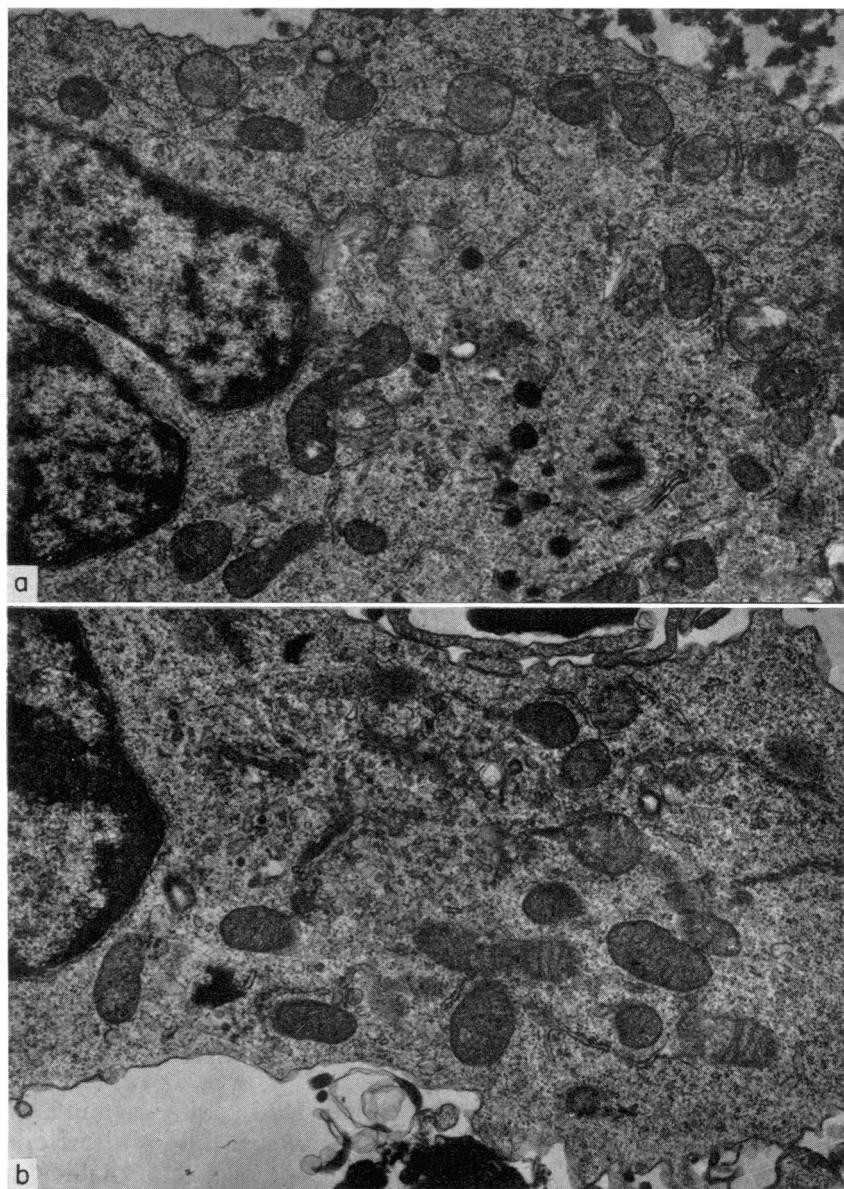


FIG. 1. Transformed lymphocyte induced by: (a) anti-allotype serum, and (b) phytohaemagglutinin. Electron micrograph, $\times 15,000$.

RESULTS

EFFECT OF AAS ON LYMPHOCYTES IN TISSUE CULTURE

Transformation of peripheral blood lymphocytes from Ab4,4, Ab4,5 or Ab5,5 rabbits grown in tissue culture in the presence of the appropriate specific AAS was consistently obtained. There was considerable variation in the extent to which lymphocytes from different rabbits responded to the same antiserum (or to PHA). Preliminary experiments showed that a maximal response occurred when the cells had been cultured for approximately 64 hours. The degree of stimulation was directly related to the concentration of AAS. Large doses of AAS, however, tended to inhibit transformation.

DNA synthesis, as measured by [^{14}C]thymidine uptake, correlated reasonably well with the percentage of lymphocytes transforming into blast cells although the former parameter was affected to some extent by the number of surviving cells in the culture at the time of harvesting while the latter was not.

Lymphocytes gave a better response to AAS when they were grown in small vials and to PHA when grown in Bijou bottles. Even when optimal conditions were used for both stimulants, there was invariably a greater response to the AAS, the uptake of thymidine being 5.8 ± 0.7 (SE) times greater in fifteen rabbits studied. It was subsequently found that a greater response could be obtained with a different preparation of PHA (Difco, PHA-M). Ling (1968) has also noted that rabbits may not respond equally well to different batches of PHA.

Lymphocytes transformed by AAS could not be distinguished from PHA stimulated cells in fixed preparations stained with May-Grünwald-Giemsa. Both cell types had prominent nucleoli and a strongly basophilic, non-granular cytoplasm (Sell and Gell, 1965). At the ultrastructural level also, appearances were closely comparable (Fig. 1a and b); there were a moderate number of mitochondria and lysosomal structures, a well defined Golgi zone and vacuoles containing electron-dense lipid material. Rough surfaced endoplasmic reticulum was not prominent in either cell type but both showed an abundance of free polyribosomes. Elongated cytoplasmic projections were observed extending from some of the blast cells. A striking feature, peculiar to the AAS cultures was the presence of an amorphous 'debris' seen in association with 87 per cent of the blast cells and only 16 per cent of the untransformed lymphocytes. It was mainly found surrounding the blast cells but material which had probably been phagocytosed could be seen in intracellular vacuoles (Fig 2a and b). The origin of the debris is not known but its appearance was similar to that of antigen-antibody complexes formed between AAS and normal rabbit serum of the appropriate allotype.

EXPRESSION OF ALLOTYPIC MARKERS IN RABBITS SUPPRESSED BY AAS

Four groups of rabbits were compared: (1) homozygous Ab4,4; (2) homozygous Ab5,5; (3) heterozygous Ab4,5; and (4) allotypically suppressed animals which were the progeny of matings between homozygous Ab5,5 bucks and homozygous Ab4,4 does having high titre antibodies to Ab5 immunoglobulin induced by immunization with Ab5 coated *P. vulgaris* prior to conception. The groups had seven, four, fifteen and fifteen rabbits, respectively, the animals being 3 months to 1 year old when tested.

The concentration of serum IgG and serum immunoglobulin allotypes Ab4 and Ab5 in the four experimental groups of rabbits were compared and the results are shown in

Figs. 3–6 where the amount of each allotype is expressed as a percentage of the total concentration of IgG. Animals exposed to AAS in foetal life, with the exception of one rabbit, showed a complete suppression of Ab5 immunoglobulin and a compensatory increase in the concentration of Ab4 molecules which was almost as high as that found in normal homozygous Ab4,4 rabbits. The levels of immunoglobulin allotypes in all groups are

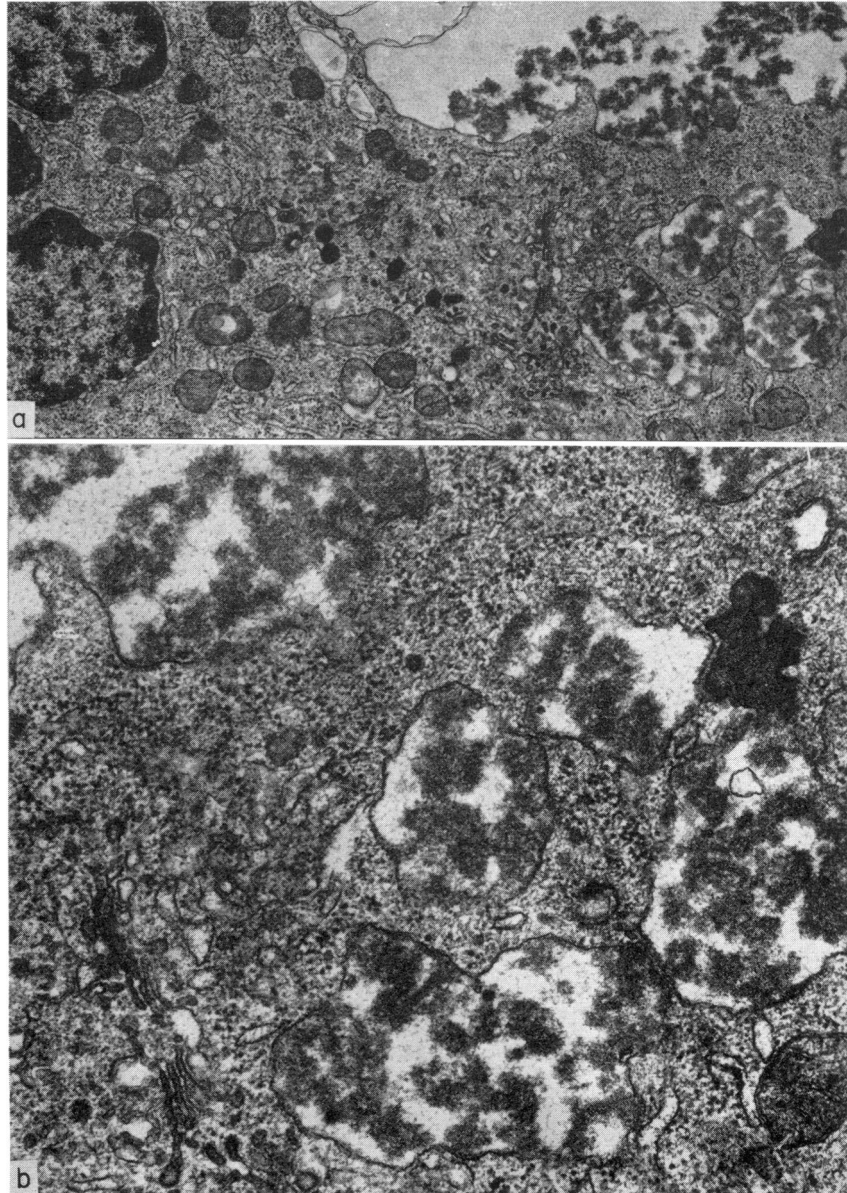


FIG. 2. Transformed lymphocyte induced by anti-allotype serum. (a) Electron micrograph showing presence of amorphous debris around the cell and within cytoplasmic vacuoles. $\times 13,200$. (b) Detail of (a). $\times 32,000$.

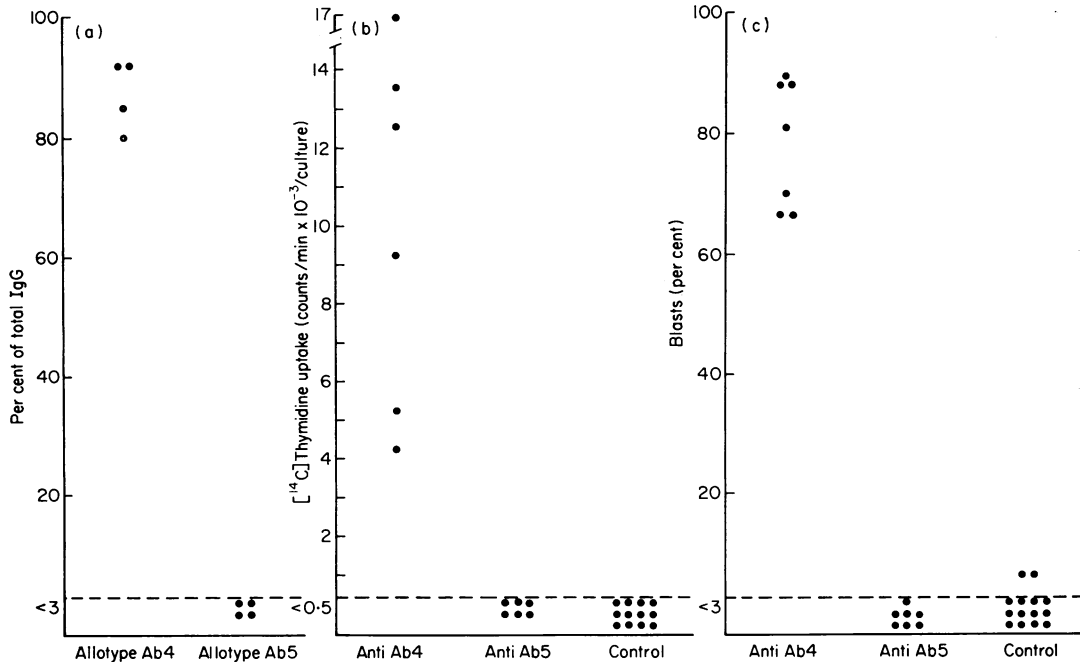


FIG. 3. Homozygous Ab4,4 rabbits: serum allotype concentration (a) and anti-allotype transformation (b and c). For the latter, each point represents the average of triplicate cultures. Anti-Ab4 cultures and their controls contained homozygous Ab6,6 normal serum. Anti-Ab5 cultures and their controls were set up in homozygous Ab4,4 normal serum. The results of both types of control are presented in the figure.

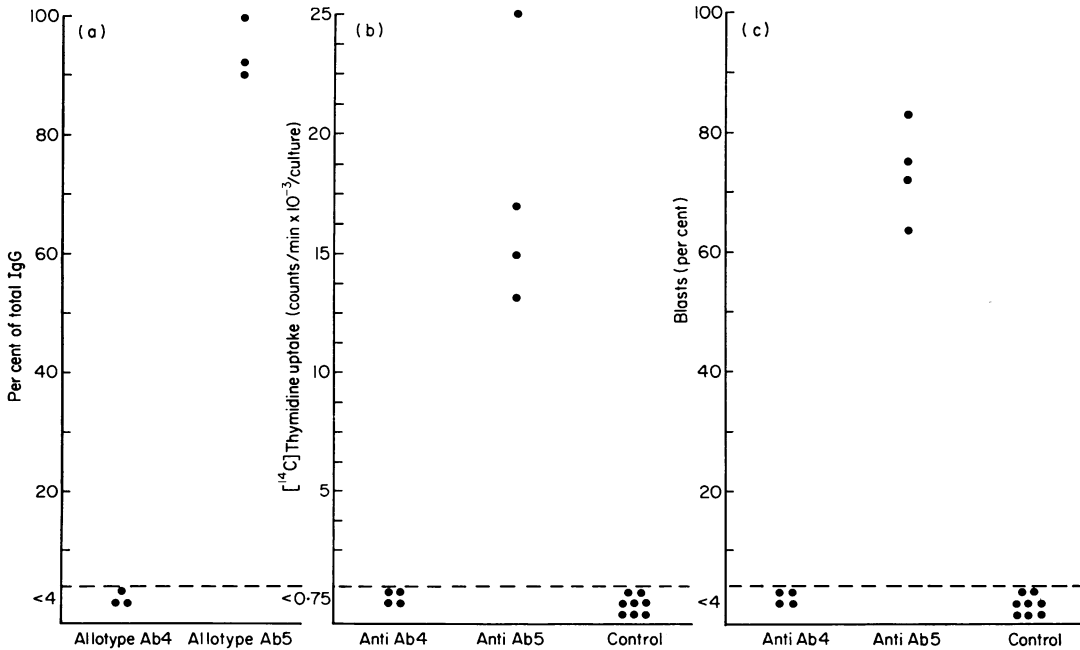


FIG. 4. Homozygous Ab5,5 rabbits: serum allotype concentration (a) and anti-allotype transformation (b and c). Details as for Fig. 4.

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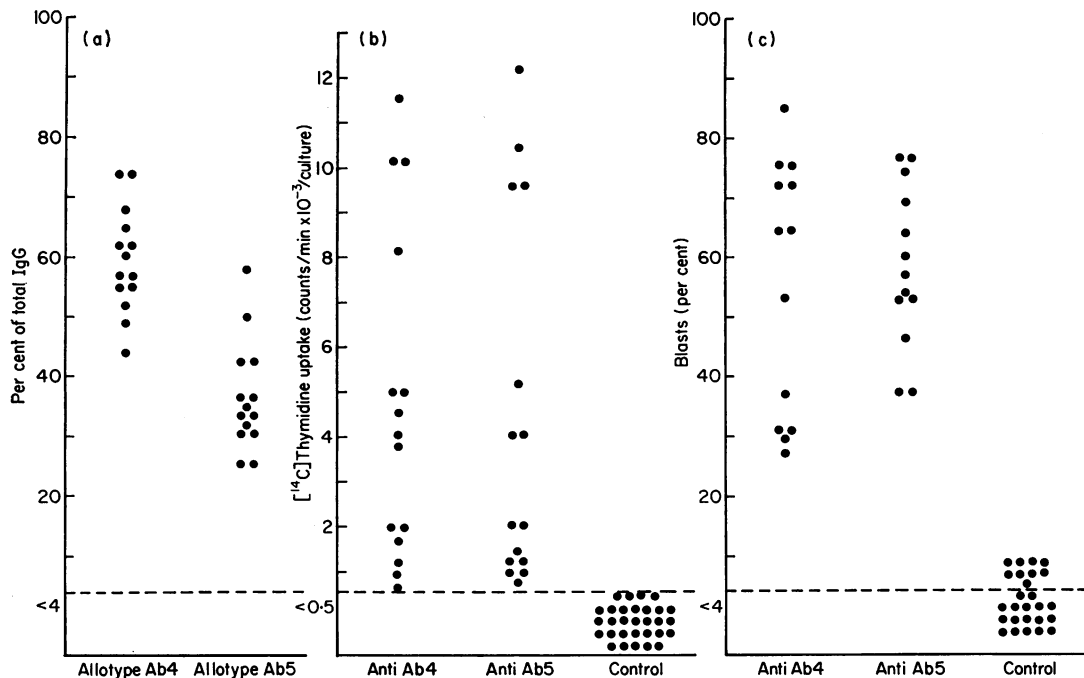


FIG. 5. Heterozygous Ab4,5 rabbits: serum allotype concentration (a) and anti-allotype transformation (b and c). Details as for Fig. 4.

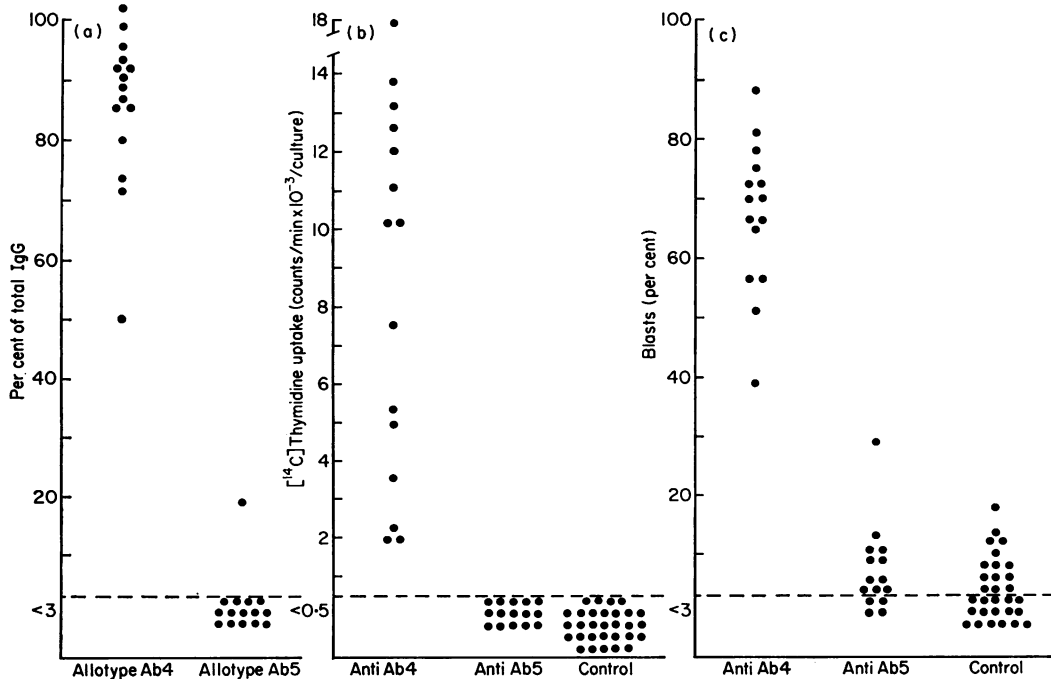


FIG. 6. Heterozygous Ab4,5 rabbits suppressed by anti-Ab5 (allotypically suppressed): serum allotype concentration (a) and anti-allotype transformation (b and c). Details as for Fig. 4.

consistent with those reported previously (Dray, 1962; Dray and Nisonoff, 1963; Bornstein and Oudin, 1964; Stemke, 1965; Mage and Dray, 1965; Kelus and Gell, 1967).

Transformation of peripheral blood lymphocytes from rabbits in the four groups was assessed from blast-cell counts and thymidine uptake and the results are shown in Figs. 3-6. Homozygous Ab4,4 and Ab5,5 rabbit lymphocytes were stimulated to a comparable extent by the appropriate AAS. The response of homozygous lymphocytes from these two groups was greater than that of the heterozygous Ab4,5 lymphocytes to either anti-serum. Lymphocytes from 'suppressed' animals, with the exception of one rabbit, were not stimulated by anti-Ab5 serum. The animal which gave a small response to this AAS was

TABLE 1
PER CENT BLAST CELL TRANSFORMATION IN CULTURES STIMULATED WITH ANTI-Ab4 AND ANTI-Ab5
AAS (MEAN \pm SE)

Group	Rabbit allotype	Anti-Ab4	Anti-Ab5
1	Ab4,4	78 \pm 4	<4
2	Ab5,5	<4	73 \pm 4
3	Ab4,5	55 \pm 6	58 \pm 4
4	Ab4,5 suppressed by anti-Ab5	67 \pm 3	<4

Significance of differences between groups (Student's *t*-test): Anti-Ab4: 1 vs 3, $P < 0.001$; 1 vs 4, $P < 0.05$; 3 vs 4, $P < 0.05$. Anti-Ab5: 2 vs 3, $P < 0.05$.

the same as that noted above showing incomplete suppression of its serum immunoglobulin allotype. The response of lymphocytes from animals in this group to anti-Ab4 was greater than that of normal heterozygous Ab4,5 rabbit lymphocytes although less than that obtained with homozygous Ab4,4 lymphocytes. The [¹⁴C]thymidine uptake by PHA stimulated lymphocytes from suppressed rabbits was below that in AAS stimulated cultures but was nevertheless about ten times that of the controls. The overall results obtained in these transformation experiments are summarized in Table 1.

DISCUSSION

The phenomenon of anti-allotype transformation has been confirmed in this study and our experience with respect to the time course and dose dependence of the reaction is in agreement with that previously reported (Sell and Gell, 1965; Gell and Sell, 1965; Sell *et al.*, 1965).

It is not known whether the cells which are transformed by AAS represent a sub-population of lymphocytes in the rabbit. In birds, at least two populations of lymphoid cells can be recognized, one which is thymus dependent and involved in cell-mediated immunity, and another which is bursa derived and implicated in the synthesis of humoral antibody (Cooper, Peterson, South and Good, 1966; Szenberg and Warner, 1967). In rabbits, the situation is less clearly defined but it has been suggested that gut-associated lymphoid tissues have a similar function to the avian bursa (Cooper, Perey, Gabrielsen, Sutherland, McKneally and Good, 1968). AAS stimulated lymphocytes may belong to the thymus related population. PHA sensitive cells in chickens are known to be thymus dependent (Greaves, Roitt and Rose, 1968) and several findings favour the view that the cells transformed by PHA in rabbits are the same as those which respond to AAS. Both agents bring about the transformation of a majority of peripheral blood lymphocytes in culture. Furthermore, our ultrastructural studies have shown that the

blast cells produced by the two mitogens are closely similar and that neither type contains significant amounts of rough surfaced endoplasmic reticulum. The presence of this structural feature in blast cells produced by agents other than specific antigens has so far only been observed in a small proportion of pokeweed mitogen stimulated human lymphocytes (Douglas, Hoffman, Borjeson and Chessin, 1967); and a similar proportion of these blasts has been found to contain cytoplasmic immunoglobulins (Greaves and Roitt, 1968). Neither human lymphocytes transformed by PHA (Greaves and Roitt, 1968) nor rabbit lymphocytes stimulated by AAS (Sell *et al.*, 1965) produced appreciable amounts of immunoglobulins. In order to obtain more direct proof of the thymic dependence of AAS responsive lymphocytes, attempts are in progress to thymectomize foetal rabbits.

One difference between the PHA and AAS stimulated cultures was the presence of an amorphous material in and around the blast cells in the latter. In the electron microscope, this debris resembled the antigen-antibody precipitates formed by mixing AAS with the appropriate normal rabbit serum. We have observed the formation of similar material occurring after a transient agglutination of lymphocytes caused by successive treatments with a specific AAS followed by a second AAS directed against the allotype of the coating immunoglobulin. It is possible that in both situations the AAS forms an immune complex with immunoglobulin markers on the surface of the lymphocyte which are presumably 'detached from' the membrane. The agglutination phenomenon and the transformation of lymphocytes by AAS are both strongly suggestive of the existence of such surface markers. The absence of 'debris' in PHA cultures may only reflect a difference in the mode of action of the two mitogens rather than in the cells responding.

Sell (1968a) found that the number of lymphocytes transformed by a given AAS is considerably smaller in heterozygous than in homozygous animals. We have confirmed this finding in the present study. Although these data suggest that there is expression of only one of two allelic specificities by individual lymphocytes they do not exclude the possibility that some lymphocytes may possess more than one specificity but not have a critical minimum number of receptor sites of each type to enable the cell to transform in the presence of a monospecific antiserum (Sell, 1967; Gell, 1967).

Phenotypic suppression of Ab5 immunoglobulins with a compensatory increase in Ab4 molecules was produced in Ab4,5 rabbits exposed to anti-Ab5 serum during the foetal period as previously described (Dray, 1962; Mage and Dray, 1965). These 'allotypically suppressed' animals were either grossly or completely deficient in peripheral blood lymphocytes capable of transforming in the presence of the AAS initially used to produce the allotypic suppression. Thus, lymphocytes from Ab4,5 animals with suppressed Ab5 immunoglobulin did not respond *in vitro* to anti-Ab5 serum. There was an increase in the number of lymphocytes transforming with anti-Ab4 relative to that found in normal heterozygous Ab4,5 rabbits; however this was not as large as that obtained with homozygous Ab4,4 lymphocytes. Where there was incomplete suppression or a partial recovery of Ab5 immunoglobulin, there was a small response by the lymphocytes to anti-Ab5 serum. The PHA transformation was not impaired in any of these animals. Since this work was completed a report has appeared in which comparable findings were described (Sell, 1968b).

The mechanism of anti-allotype suppression is still unknown. In view of the fact that such diverse cell types as those involved in antibody synthesis (Lumms *et al.*, 1967) and lymphocyte transformation are implicated, and that complete recovery may be delayed for as long as 3 years (Mage and Dray, 1965), it would seem that a relatively primitive cell is affected. It has previously been suggested (Mage and Dray, 1965) that AAS may

act on an intracellular regulatory mechanism of the type proposed by Jacob and Monod (1961) and 'switch over' the cells from one allotype to its allele. Alternatively, the AAS may eliminate cells either by a complement-mediated cytotoxic reaction or by opsonization. Preliminary experiments have indeed suggested that AAS is able to opsonize lymphocytes *in vitro*. One is tempted to draw a parallel between allotypic suppression and immunological tolerance in which specifically reactive cells can be removed by contact with the antigen in early life and from which there may be a gradual recovery.

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