Immunology, 1965, **9**, 565.

The Transformation *in vitro* of Peripheral Lymphocytes of Some Laboratory Animals

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(*Received* 20*th April* 1965)

Summary. The peripheral lymphocytes of monkeys, rabbits, guinea-pigs, hamsters, rats and mice were transformed in culture by phytohaemagglutinin and staphylococcal filtrate. The response was most marked with the peripheral lymphocytes of monkeys and rabbits and least marked with rat and mouse lymphocytes. The response occurred earlier with rabbit and guinea-pig than human lymphocytes.

The short life span *in vitro* of rat and mouse lymphocytes adversely affected their response. The tritiated thymidine uptake of the stimulated lymphocytes of the various species was also limited by their rate of survival over the culture period.

Attempts to obtain antigen-specific stimulation of peripheral rabbit and guinea-pig lymphocytes were unsuccessful.

INTRODUCTION

Peripheral blood lymphocytes of human origin may be non-specifically stimulated *in vitro* to undergo transformation into blast forms with stimulants such as phytohaemagglutinin, staphylococcal filtrate or streptolysin S (see review by Robbins, 1964). In contrast to the many reports on the stimulation of human peripheral lymphocytes only a few reports on the *in vitro* stimulation of lymphocytes of animal origin have been published, and the findings in these reports are often conflicting. Because of the lack of consistent data on animal lymphocytes, the experiments reported in the present paper were designed to test the transforming capacity of the peripheral lymphocytes of some common laboratory animals to two of the stronger non-specific stimulants, namely phytohaemagglutinin and staphylococcal filtrate. In addition, since peripheral lymphocytes from sensitized human donors are known to have the ability to undergo blast transformation when cultured in the presence of specific antigen, the present report also describes preliminary experiments in which the lymphocytes from immunized animals were cultured in the presence of specific antigen.

MATERIALS AND METHODS

Animals

Primates. Four species of primates were used: (i) twelve Macaca mulatta (rhesus monkeys) were females aged 6–16 years, (ii) one *Papio anubas* was a young mature female, (iii) one *Perodicteus potto* was a pregnant female, and (iv) one *Erythrocebus pattas* was a young female. Vitamin B_{12} deficient monkeys showed the symptoms previously described (Oxnard, 1964).

Other animals. Rabbits (sixty), guinea-pigs (forty-one), hamsters (seven) and rats (eighteen) were stock laboratory animals obtained from various sources. Mice were of the Strong A cancer strain.

Blood samples

Human blood was obtained by venepuncture. Monkeys were bled by venepuncture after injection of phencyclidine. Rabbits were bled from the marginal ear vein. Guinea-pigs, hamsters and rats were anaesthetized with ether and bled by heart puncture. Mice were bled from the retro-orbital venous sinus. For each experiment a pool of blood from about a dozen mice of the inbred strain was used.

Immunization schedules

Eight guinea-pigs were each injected in the foot-pads with 0·125 ml of an emulsion of Armour's bovine γ-globulin (BGG) in an equal volume of Freund's complete adjuvant (Difco) containing 0·25 mg of tubercle bacilli and 2·5 mg of BGG. Two rabbits each received 0·25 ml of the same emulsion in each foot-pad. Nine guinea-pigs and two rabbits were each injected in the rear foot-pads with a total of 0·1 ml of bacillus Calmette–Guerin (Glaxo) in saline.

Markedly positive delayed skin reactions (24 hour maximum) were elicited with 100 International Units of purified protein derivative (PPD), in all the immunized animals.

In addition four rabbits received intravenous courses of bovine serum albumin (Armour) or human γ-globulin (Cohn Fraction II, prepared by Mr R. Stokes) as described by Dutton and Eady (1964).

Blood leucocyte preparations

A technique based on that of Coulson and Chalmers (1963) was found to be the most satisfactory for routine purposes. Blood was drawn into siliconed containers and gently defibrinated. After mixing with a half volume of 3 per cent gelatin in saline (fine grain pig skin gelatin, British Glue and Chemicals Ltd) the blood was poured into clean screw-cap bottles and allowed to sediment for 1–2 hours at 37°. The leucocyte-rich supernatant fluid was then transferred to fresh bottles. Large volumes of gelatin ($1\frac{1}{2}$: 1 volume of blood) were required to sediment hamster erythrocytes. Some samples of monkey blood were collected in heparin and the erythrocytes sedimented with dextran as previously described for human blood (Ling, Spicer, James and Williamson, 1965). The defibrination-gelatin technique always gave good yields of viable lymphocytes enriched in relation to the other leucocytes (commonly over 90 per cent of the leucocytes were lymphocytes in rabbit preparations) but erythrocyte contamination ranged from 5 to 30 erythrocytes per lymphocyte. The heparin–dextran technique yielded leucocytes with a differential count similar to that of the original blood. Erythrocyte contamination was low (one to two erythrocytes per lymphocyte). The dextran method was not applicable to blood of rabbits or guinea-pigs since their plasma clotted after a short period of incubation with culture medium, enmeshing the cells in the clot.

Lymphocyte cultures

Cultures were set up in bijou bottles, each containing a minimum of $0{\cdot}5 \times 10^6$ leucocytes, stimulant (when added) and Eagle's medium (containing 10 per cent tryptose and penicillin and streptomycin) to a final volume of 3 ml. The bottles were tightly capped and incubated at 37°. At the end of the incubation period, the cells were spun down, smears prepared and stained as previously described (Ling *et al.*, 1965). The stimulants used

were phytohaemagglutinin M (PHA) the Difco product (0·03 ml/culture) and staphylococcal filtrate (SF, 0·5 ml/culture). Tuberculin, purified protein derivative (PPD) was employed as previously described (Ling and Husband, 1964).

Viability

Viability of cells was assessed by dye-exclusion as described by Black and Berenbaum (1964).

Autoradiography

Tritiated thymidine (thymidine (methyl)-T 16·1 mc/mg, Amersham) was added (1 μc/culture) to some cultures 2 hours before harvesting. Shorter periods of incubation were used for human and monkey cells when pulse labelling followed by grain counts was intended. Methanol-fixed smears were washed, layered with Kodak AR10 stripping film and exposed for 1–3 days. After development with D19B developer the smears were stained with Giemsa, according to the method of Gude, Upton and Odell (1955). In some experiments tritiated thymidine uptake measured autoradiographically was supplemented by measurement of the overall uptake of [^{14}C]thymidine by duplicate cultures following the technique of Dutton and Eady (1964). The same technique was used to measure the DNA synthesis of rabbit lymphocytes stimulated with antigens.

Fluorescent conjugates

The γ-globulin fractions of sheep anti-rabbit serum proteins, guinea-pig anti-rabbit γ-globulin and rabbit anti human γ-globulin were conjugated with fluorescein isothiocyanate by the method of Coons and Kaplan (1950). The first antiserum reacted with many rabbit serum proteins while the second was specific for γ-globulin when tested by immunoelectrophoresis. Both conjugates stained plasma cells in sections of rabbit lymph nodes. Smears of leucocytes were prepared and treated with fluorescent antibodies by the method of Van Furth (1964).

RESULTS

PRIMATES

Blood lymphocytes from twelve rhesus monkeys were tested and ten responded markedly to both stimulants to a degree comparable to that obtained with human lymphocytes (Table 1). Lymphocytes from the two negatives were tested only with SF and the failure may have been due to a faulty batch of SF. No difference was discernible between the response of lymphocytes of normal and of the vitamin B_{12} deficient monkeys, nor was the reaction modified by the addition of vitamin B_{12} to the cultures.

Lymphocytes transformed by PHA were regularly smaller and with a less highly basophilic cytoplasm than those formed with SF. The transformed cells closely resembled human transformed lymphocytes (Fig. 3a and b). Most monkey lymphocyte cultures contained 0·5 ml of leucocyte-rich plasma containing $0{\cdot}5$–$1{\cdot}8\times10^6$ white blood cells in a total volume of 3 ml of culture. One of the monkeys had a high granulocyte count and this culture contained 8×10^6 white blood cells. Blood lymphocyte cultures from this monkey were normally activated by SF, but were relatively unaffected by PHA. The rate of DNA synthesis of transformed monkey lymphocytes as judged from ^{3}H-thymidine uptake by autoradiography was about three times that of rabbit transformed cells and about half that of human (Fig. 3d, e and f).

TABLE 1

PERCENTAGE OF BLAST CELLS PRESENT IN DIFFERENT SPECIES AFTER VARIOUS CULTURE PERIODS

Species	Stimulant	Days of culture				
		1	2	3	4	5
Human	None	0	0	0	0	5
	SF	0	7(5)	48(41)	48(40)	73(48)
	PHA	0	10(8)	38(64)	90(54)	0
Rabbit	None	0	0	0	4(1)	1
	SF	2(1)	50(39)	50(40)	20(7)	13(8)
	PHA	4	8(2)	39(29)	20(5)	15(5)
Rat*	None	0·2	0·2	0·2	0	
	SF	0·6	0·6	19	0	
	PHA	0·4	0	18·2(3·4)	0	
Guinea-pig*	None	0	0	0·2	0	
	SF	0·5	2(1)	1·6	0	
	PHA	30(3)	35(8)	0	0	
Mouse*	None	0	0	0		
	SF	<1	0	0		
	PHA	<1	0	0		
Hamster	None	0	0	0		
	SF	0	7	0		
	PHA	0	0	0		
Monkeys						
Macaca mulatta	None	0	0	0	0·2(0·2)	
	SF	0	3·6(0·8)	29(19)	94(48)	
	PHA	0	3·6(1·8)	27(22)		
Papio anubas	None	0	0	0	0·4	0
	SF	0	0	2·6(0·8)	5·8(2)	7·6(0·6)
	PHA	0	0	0	0	2·4(0·4)
Perodicteus potto	None	1	2	3·2(1·6)	8·3(3)	
	SF	0·5	3·8(2·4)	20·4(0·7)	24·5(1·5)	
	PHA	0	6·6(5)	14·5(2)	43(3)	

Principal entry = Per cent blasts (morphological classification); entry in parentheses = per cent cells autoradiographically positive after incubation with [^{3}H]thymidine.

* Received [^{3}H]thymidine for 24 hours prior to harvesting instead of the routine 2 hours.

Of the single specimens of the other primates tested, baboon lymphocytes showed some blast formation, although this occurred later and only 7–8 per cent of the lymphocytes were affected. *Perodicteus potto* preparations were unusual in showing spontaneous blast formation (Fig. 3c) and large numbers of granulocytes containing large clear inclusions persisted for 5 days. *Erythrocebus pattas* lymphocytes from a single blood sample were not transformed by either stimulant.

RABBITS

Marked stimulation of rabbit lymphocytes was regularly obtained with both SF and PHA (Table 1). Large blast-like cells with prominent nucleoli and basophilic cytoplasm were formed (Fig. 3k). Although the actual percentage of cells stimulated varied from animal to animal there were usually not fewer than 35 per cent blasts at the peak of transformation. Tritiated thymidine uptake was closely related to size distribution. An increase in the size of lymphocytes appeared before DNA synthesis began in stimulated cultures (Fig. 1, 24 hours) and continued until most of the transformed lymphocytes ceased to synthesize DNA (Fig. 1, 96 hours) The nuclei of lymphocytes in unstimulated cultures were less than 8 μ diameter until the 4th day when occasional large thymidine positive

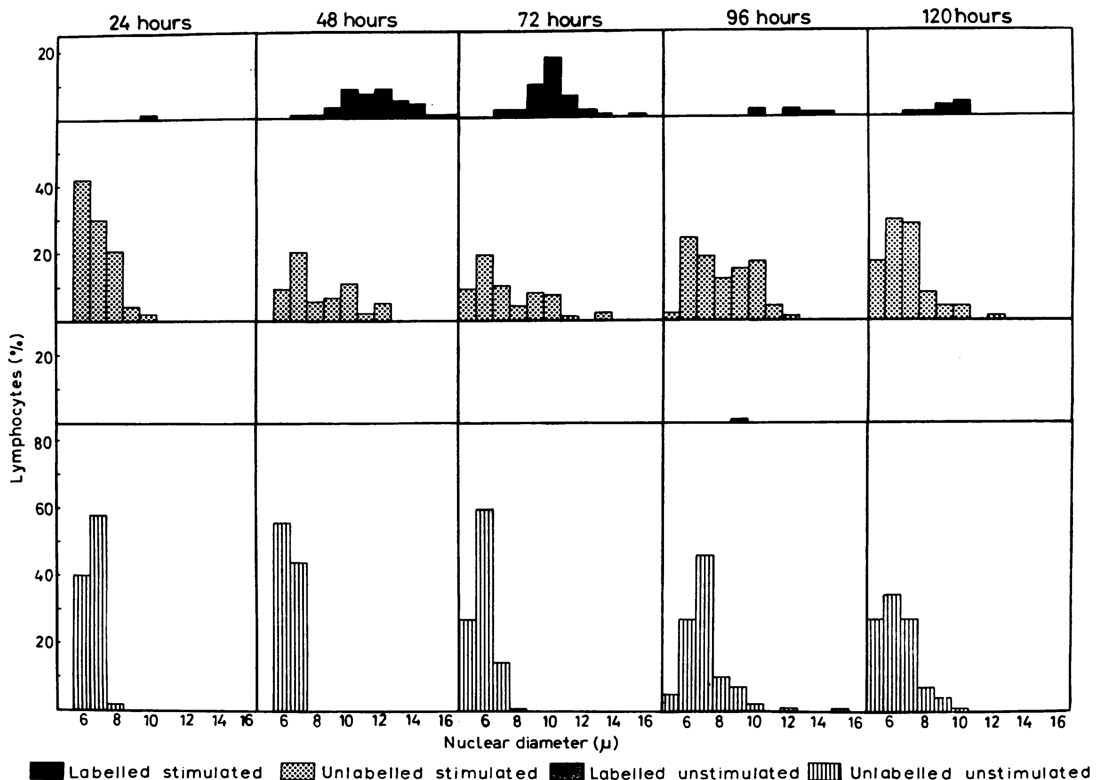

FIG. 1. Frequency distribution of cultured rabbit peripheral lymphocytes in relation to nuclear diameter. Stimulated cultures (which received SF) and control cultures (no stimulant) are classified as labelled or unlabelled on the basis of autoradiography under the standard conditions after incubation with [^{3}H]thymidine. Nuclear size was estimated from two perpendicular measurements of the nuclear diameter.

cells appeared. The lymphocyte population responded to stimulation in a markedly asynchronous manner in terms of thymidine uptake. After a lag period of 24 hours, DNA synthesis proceeded to a maximum between 48 and 72 hours (Table 2). The sequence was similar to that in human lymphocyte cultures (compare Figs. 1 and 2) but took place over a shorter time interval. The drop in thymidine uptake after longer periods of culture was accompanied by a drop in the percentage of viable cells (Table 3). Stimulation was obtained at a wide range of cell concentration, from $0{\cdot}15\times10^6$ to 5×10^6 leucocytes per ml.

TABLE 2

RATE OF DNA SYNTHESIS AFTER VARIOUS INTERVALS OF CULTURE OF RABBIT LYMPHOCYTES STIMULATED WITH SF

	Culture period (hours)				
	24	48	72	96	120
Grains per 100 lymphocytes	–	895	758	90	138

Cultures were incubated with [^{3}H]thymidine for 2 hours prior to harvesting; grain counts made on autoradiographs.

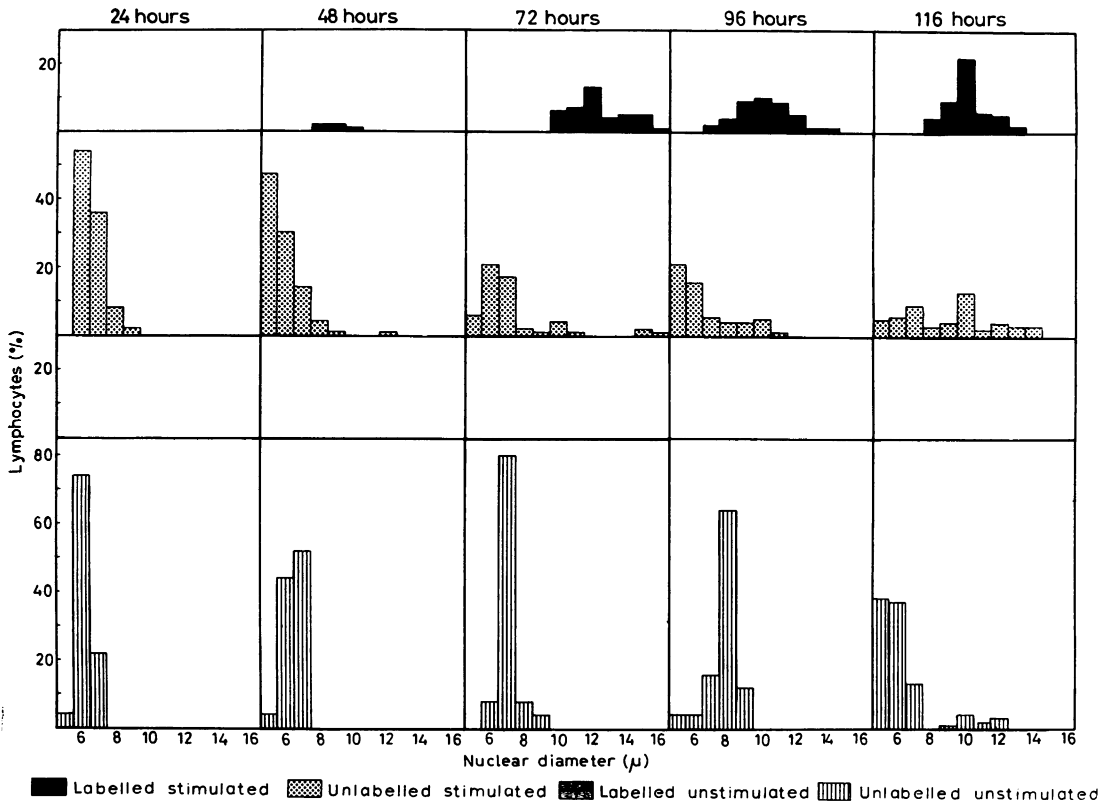

FIG. 2. Frequency distribution of human peripheral lymphocytes in relation to nuclear diameter. Stimulated cultures (which received SF) and control cultures (no stimulant) are classified as labelled or unlabelled on the basis of autoradiography under the standard conditions after incubation with [^{3}H]thymidine. Nuclear size was estimated from two perpendicular measurements of the nuclear diameter.

The use of higher cell concentrations was limited by acid production during the culture period, but this was relatively low in the granulocyte-poor leucocyte preparations used.

No cells containing γ-globulin were detected in smears made at 3–6 days, using fluorescein-conjugated guinea-pig anti-rabbit γ-globulin. Lymphocytes, both small and transformed, were positive in the immunofluorescence test to varying degrees after treatment

TABLE 3

VIABILITY OF CULTURED LYMPHOCYTES

Species	Hours of culture			
	0	24	48	72
Rabbit	100	68	54	39
Guinea-pig	98	37	33	24
Rat	96	48	19	5
Mouse	90	27	5	0
Hamster	90	84	17	5

The figures refer to the percentage viable cells in the preparation as determined by dye-exclusion.

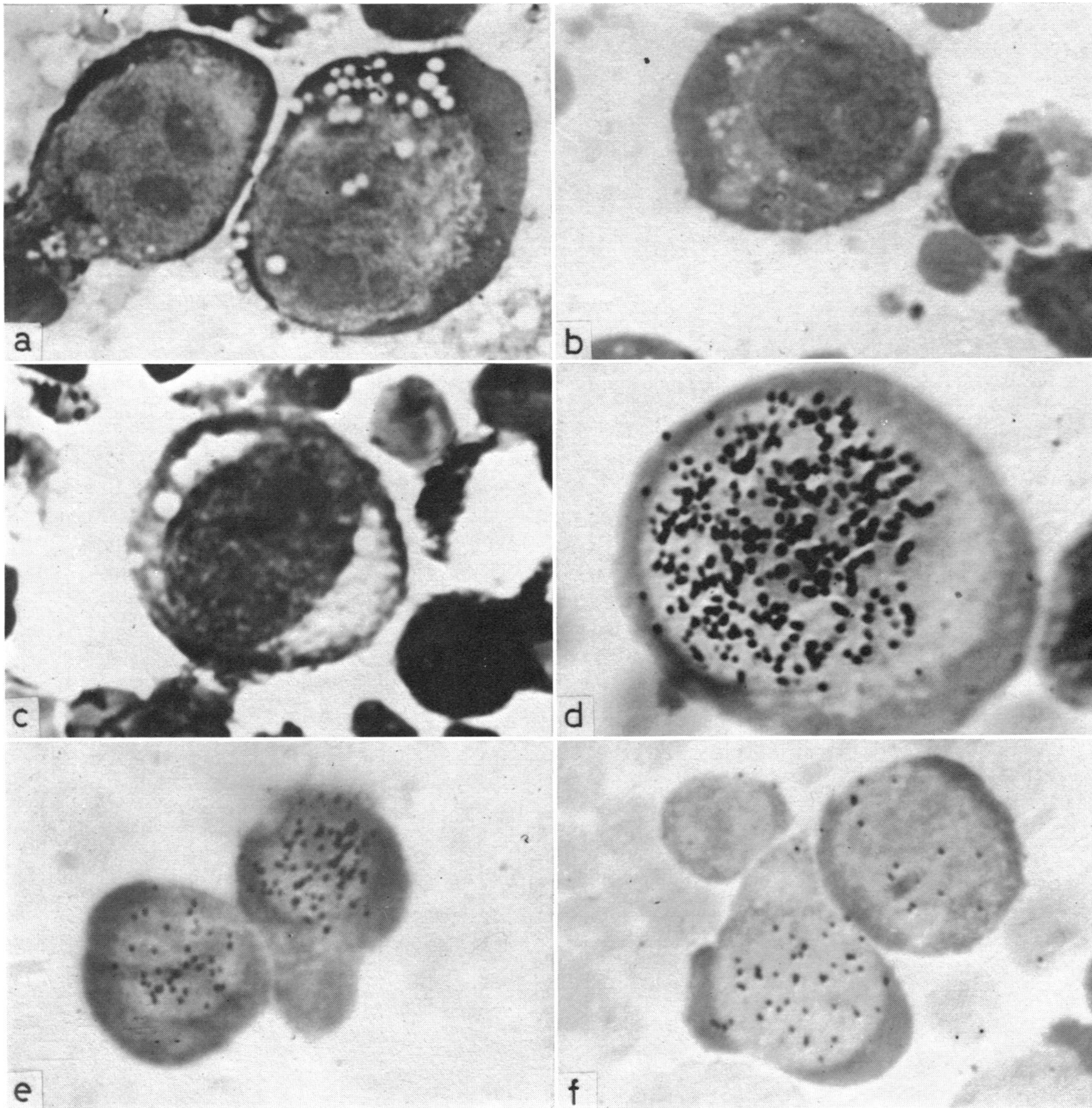

FIG. 3. Transformed peripheral lymphocytes from various species: (a) Human, stimulant SF. × 2000. (b) Rhesus monkey, stimulant SF. × 2000. (c) *Perodicteus potto*, blast cell from culture with no stimulant. × 2000. (d) Human, stimulant SF. Autoradiograph after incubation with [^{3}H]-thymidine. × 3000. (e) Rhesus monkey, stimulant SF. Autoradiograph after incubation with [^{3}H]-thymidine. × 1500. (f) Rabbit, stimulant SF. Autoradiograph after incubation with [^{3}H]-thymidine. × 1500.

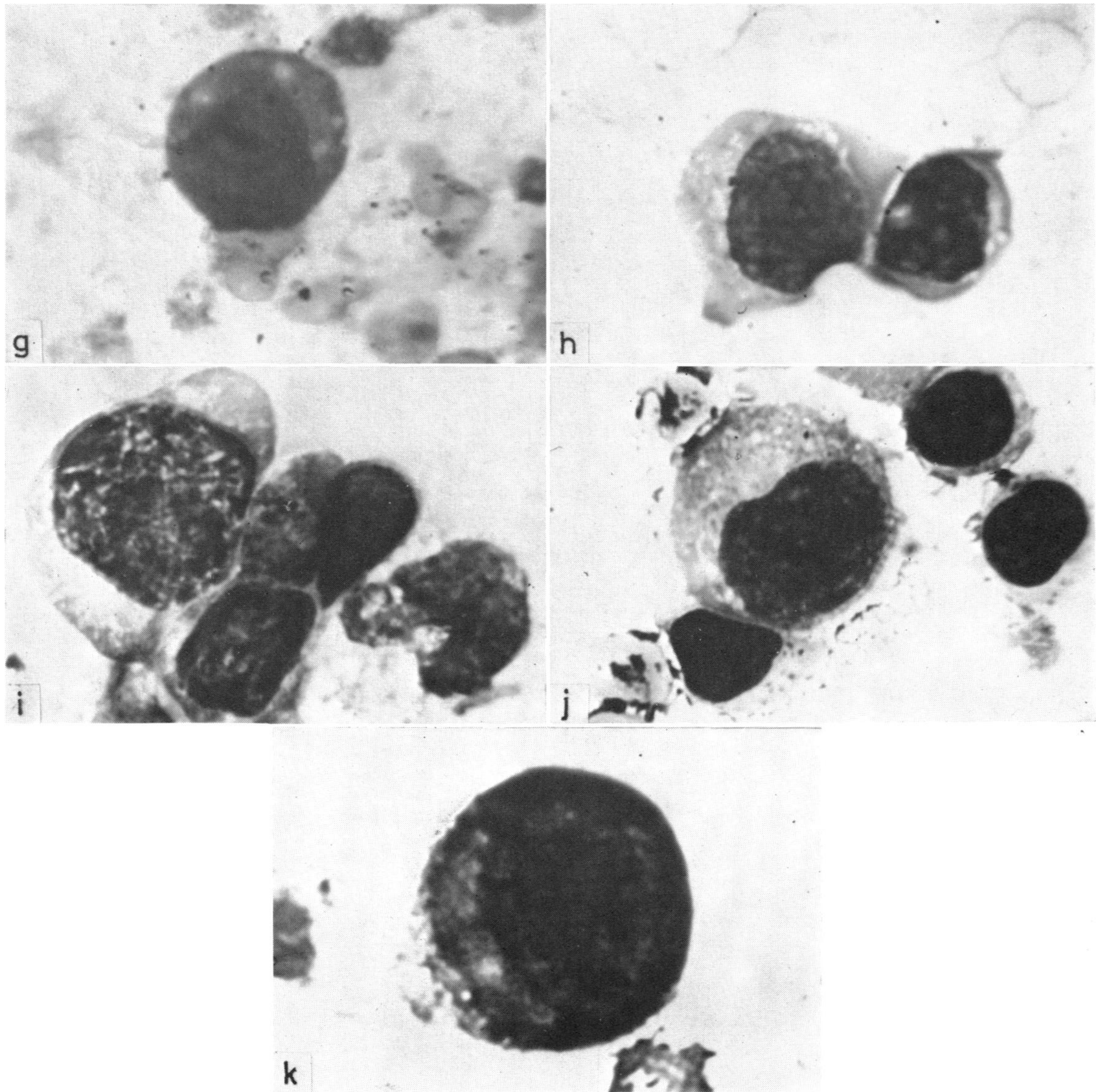

FIG. 3 (*continued*). (g) Mouse, stimulant PHA. Transformed lymphocyte amongst cell debris. × 1500. (h) Hamster, stimulant SF. × 1500. (i) Guinea-pig, stimulant PHA. × 2000. (j) Guinea-pig, stimulant SF. × 2000. (k) Rabbit, stimulant PHA. × 2000.

with the conjugate to whole rabbit serum. Peripheral lymphocytes from rabbits immunized with bovine albumin or human γ-globulin were not stimulated by these antigens *in vitro* under these conditions (Table 4 exemplifies the findings). Lymphocytes from tuberculin-positive rabbits were not stimulated *in vitro* by a range of concentrations of PPD (10–250 μg/ml) when examined after 2–5 days in culture.

Table 4

Effect of antigen on the DNA synthesis of cultured rabbit peripheral lymphocytes

Antigen used		[^{14}C]thymidine uptake (counts/min)
For immunization	For the *in vitro* stimulation	
Bovine serum albumin (BSA)	None	100
	BSA	47
	HGG	71
Human γ-globulin (HGG)	None	21
	BSA	12
	HGG	28

Cultures (set up in quadruplicate) contained 2.mg/ml of the antigen under test and were harvested at 48 hours after incubation with 0·12 μc of [^{14}C]thymidine during the preceding 24 hours. Each culture (vol. 3 ml) contained 5×10^6 white blood cells which had been washed and suspended in medium containing 15 per cent normal rabbit serum and the antigen under test.

GUINEA-PIGS

Guinea-pig lymphocytes were on many occasions activated by PHA and sometimes by SF. Blasts were present at 24 and 48 hours, but less frequently at 72 hours. The marked variations of response which occurred on different occasions were not dependent on the animal used since some animals were tested on several occasions with different results. Leaving the blood at 37° for some hours before processing appeared to improve the results as did increasing the cell concentration to 3×10^6 per culture. The small number of blast cells formed with SF were typical large blasts whereas those formed in larger numbers with PHA were frequently less typical and appeared to be arrested in development in a transitional stage (Fig. 3). More convincing proof of the transformation was obtained from autoradiographs prepared from cultures incubated for 24 hours with ^{3}H-thymidine.

Autoradiographically positive cells which had subsequently undergone degeneration were then frequently seen. Lymphocytes from tuberculin positive guinea-pigs were not stimulated *in vitro* by PPD (10–250 μg/ml) when examined after 1–4 days in culture.

RATS AND MICE

With mouse and to a lesser extent rat lymphocytes, the viability of the lymphocytes over the culture period was an extremely critical factor (see Table 3). Between 10 and 20 per cent of the rat lymphocytes remaining after 3 days were transformed with PHA and SF used in the usual concentrations, whereas control cultures showed less than 1 per cent transformation (Table 1). Employing $\frac{1}{2}$–3 times the usual concentrations of PHA made no difference to the response, but with SF the response showed an optimum at 0·5 ml per 3 mμ culture. In mouse lymphocyte cultures changing the cell concentration from $\frac{1}{2}$ to 4 times that usually used, changing the proportions of serum and Eagle's medium, culturing

in calf serum instead of autologous serum and using different concentrations of stimulant made no appreciable difference to the survival time. Occasional transformed cells were regularly found amongst the cell debris after 24 and 36 hours in mouse cultures (Fig. 3g) but not after longer periods of culture. It was also observed that the erythrocytes in mouse cultures underwent lysis after short periods of culture.

HAMSTER

Typical large blasts were found in smears prepared after 2 days' culture with SF (Table 1, Fig. 3h) but blasts were not found in cultures containing PHA. Hamster cultures contained less autologous serum than usual in view of the larger volume of 3 per cent gelatin required to sediment the erythrocytes.

MORPHOLOGY OF THE TRANSFORMED CELLS (Fig. 3a–k)

Most human, monkey, rabbit and guinea-pig lymphocytes cultured for short periods in the absence of stimulant had a nuclear diameter of 5–7 μ, whereas rat and mouse lymphocytes were slightly smaller (nuclear diameter 4–6 μ). The transformed lymphocytes formed after stimulation showed in all species enlarged nuclei, cytoplasmic basophilia and frequently prominent nucleoli. A perinuclear clear zone was usually present. Most transformed cells contained cytoplasmic vacuoles; these occurred more frequently with SF than PHA stimulation and were more prominent in human and monkey cells than in transformed cells from other species (Fig. 3a, b and c). Transformed cells formed in cultures containing SF were usually larger than those formed in cultures containing PHA, e.g. the nuclei of rabbit lymphocytes transformed with PHA were mainly between 8 and 12 μ in diameter whereas those transformed with SF were in the range 8–15 μ. The corresponding values for rat transformed lymphocytes were 8–11 and 8–13 μ respectively. In cultures stimulated with PHA, agglutination of cells invariably occurred (Fig. 3i) whereas cells were not agglutinated by SF (Fig. 3j) Degenerate cells were also more frequently found in cultures containing PHA than in those containing SF.

DISCUSSION

Various brief reports in the literature concerning the transformation of animal lymphocytes have been inconclusive and often contradictory. Thus Pearmain and Lycette (1963) reported blast formation from guinea-pig peripheral lymphocytes after 4 days incubation with PHA, whereas Marshall and Roberts (1963) were not able to obtain stimulation of lymphocytes of C57 mice, guinea-pigs, rabbits or rats with PHA; the lymphocytes of one monkey responded while those of another monkey did not. Aspegren and Rorsman (1964) found that the lymphocytes of tuberculin-positive guinea-pigs did not respond *in vitro* to tuberculin, but responded to PHA. Schrek (1959) also reported a positive response of guinea-pig, but not of rat lymphocytes to PHA (Schrek and Rabinowitz, 1963). Nichols and Levan (1962) obtained good chromosome preparations from the peripheral blood of rabbits, guinea-pigs, rats and mice after 3–5 days' culture with PHA. Ohnuki, Awa and Pomerat (1962) found little, if any, stimulation of monkey lymphocytes with PHA, but reported that Sasaki and Sasaki (1962, quoted by Ohnuki *et al.*) had obtained stimulation of fox, mink, pig and rabbit, but not rat lymphocytes. Sanders and Humason (1964) were able to obtain chromosome preparations from the peripheral lymphocytes of some monkeys.

Our results suggest that the lymphocytes of most species can be induced to transform into blast cells, within the limits imposed by cell survival under the culture conditions. The many instances of a more clear-cut response to stimulation with SF rather than PHA are probably due to the mild toxic effect of commercial preparations of PHA. Degenerate lymphocytes are often seen in cultures of human or rabbit lymphocytes containing PHA but not so frequently in those containing SF. It is possible that the leucoagglutinin present in PHA, which is thought to be the active stimulating agent, itself damaged the cell membrane.

Two factors probably influenced the extent to which [^{3}H]thymidine was taken up by the lymphocytes in the cultures, viz. the normal functioning of the cells and the coincidence of the DNA synthesizing period with the time of incubation of the cells with [^{3}H]thymidine. The small number of human transformed lymphocytes not synthesizing DNA may have reached a true post-synthetic (G2) period of the cell cycle. The much higher percentage of transformed cells autoradiographically negative in rat lymphocyte cultures had evidently ceased to function normally within the mitotic cycle under the *in vitro* conditions. In agreement with Schrek (1959) rat and mouse lymphocytes were found to be short-lived *in vitro*. The earlier maximum of DNA synthesis with animal cells was largely due to premature cessation of activity, but there was also evidence of an earlier response to stimulation, particularly in guinea-pigs.

Human peripheral lymphocytes may be stimulated in an immunospecific manner. Particular interest attaches to reports of immunospecific stimulation by tuberculin of the peripheral lymphocytes of tuberculin-positive persons (Pearmain, Lycette and Fitzgerald, 1963; Cowling, Quaglino and Davidson, 1963) and to the activation produced by mixing the leucocytes of two unrelated individuals (Bain, Vas and Lowenstein, 1964).

We have not so far obtained any evidence of immunospecific stimulation of peripheral animal lymphocytes but there are good reasons for believing that under the right conditions immunospecific effects would be attainable. Chapman, Parkhouse and Dutton (1964) obtained specific as well as non-specific stimulation of rabbit lymph node and spleen cells with various agents although they found little stimulation of peripheral lymphocytes. Moynihan, Jackson and Hardy (1965) found transformed and mitotic cells in mixed peripheral leucocyte cultures from squirrel monkeys. Sell and Gell (1965, unpublished work) have obtained specific stimulation of peripheral rabbit lymphocytes with iso-antibody to allotypic γ-globulin. These are all indications that with improved culture conditions a wider range of activity of peripheral animal lymphocytes might be revealed.

ACKNOWLEDGMENTS

We wish to thank Dr K. W. Walton for advice and encouragement and Josephine Davies and Jenny Lowe for technical assistance. Dr R. Millward-Sadler assisted in some experiments.

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