Clin. exp. Immunol. (1968) **3**, 393–412.

THE EFFECT OF PHYTOHAEMAGGLUTININ AND OTHER LYMPHOCYTE MITOGENS ON IMMUNOGLOBULIN SYNTHESIS BY HUMAN PERIPHERAL BLOOD LYMPHOCYTES *IN VITRO*

M. F. GREAVES AND I. M. ROITT

Department of Immunology, Middlesex Hospital Medical School

(*Received* 11 *December* 1967)

SUMMARY

Amino acid incorporation techniques and immunofluorescence have been used to investigate the effect of mitogenic substances on immunoglobulin synthesis by human peripheral blood lymphocytes *in vitro*. Radioelectrophoresis, radioimmunoelectrophoresis and controlled immunological precipitation methods suggest that only a small amount of immunoglobulin is synthesized in the culture system used. Immunofluorescent staining of fixed cell preparations showed that during the first 24 hr in culture only a small percentage of cells reacted positively for immunoglobulin; after 24 hr these cells were no longer demonstrable. This suggests that the small amount of immunoglobulin detected was synthesized during the first few hours in culture by these cells, having the morphological appearance of medium lymphocytes. The slight enhancement of immunoglobulin synthesis obtained in one experiment with phytohaemagglutinin (PHA) probably occurred within this same cell type since after 24 hr *in vitro* no cells in the transformed cultures could be stained by the fluorescent anti-immunoglobulin. Fixed preparations of blast cells obtained by stimulation with anti-lymphocytic serum and staphylococcal filtrate also gave negative reactions. However, using a staining technique with suspensions of viable cells, it was possible to demonstrate positive staining for immunoglobulins with PHA stimulated cells as previously described by Ripps & Hirschhorn (1967). A number of controls suggest that this reaction depends upon the presence of exposed immunoglobulin groups or markers on the cell surface and that intracytoplasmic staining is the result of endocytosis of conjugate.

In contrast with the negative results obtained with PHA-transformed blasts, a small percentage of lymphocytes from cultures stimulated by pokeweed or tuberculin reacted positively when fixed preparations were stained with conjugated anti-immunoglobulin.

Correspondence: Professor I. M. Roitt, Department of Immunology, Arthur Stanley House, Middlesex Hospital Medical School, London, W.1.

INTRODUCTION

Phytohaemagglutinin (PHA), an extract of the kidney bean *Phaseolus vulgaris*, is a complex substance capable of stimulating growth and cell division of human peripheral blood lymphocytes *in vitro*. Since the original description of this transformation by Nowell (1960), various morphological and biochemical aspects of this response have been reported (Robbins, 1964). A number of studies using immunofluorescent, radioelectrophoretic and immunological precipitation techniques, have suggested that lymphocyte transformation may involve stimulation of immunoglobulin and specific antibody synthesis (Elves *et al.*, 1963; Hirschhorn *et al.*, 1963; Forbes, 1965; Cooperband *et al.*, 1966; Parenti *et al.*, 1966; Turner & Forbes, 1966; Ripps & Hirschhorn, 1967). Other workers using similar techniques have, however, been unable to confirm their findings (Balfour, Cooper & Alpen, 1965; Sell, Rowe & Gell, 1965; Epstein, 1966).

Peripheral blood taken from immunized animals appears to contain cells which synthesize immunoglobulin and specific antibody (Hullinger & Sorkin, 1963; Landy, Sanderson & Jackson, 1965). Van Furth, Schuit & Hijmans (1966), have shown that a small percentage of the lymphoid cells in human peripheral blood contain immunoglobulin as detected by the fluorescent antibody technique and that under the appropriate conditions lymphocyte cultures will incorporate radioactive amino acids into immunoglobulin. The high cell concentration and the short term incubation procedures used by Van Furth *et al.* (1966) contrast with the conditions normally employed for studying lymphocyte transformation where the number of cells must be far lower to ensure cell survival over the 3-day culture period. For this reason it might be anticipated that the small amount of immunoglobulin produced by untreated peripheral blood lymphoid cells would be difficult to detect in the lymphocyte transformation system. If PHA stimulates immunoglobulin production as suggested by Ripps & Hirschhorn (1967) then such synthesis might be detectable, although an increase in immunoglobulin could arise either from the same number of cells synthesizing protein at a faster rate or from the recruitment of a greater number of lymphocytes. Immunofluorescent staining of many or all transformed lymphocytes for immunoglobulin would favour the latter possibility. One other factor which may complicate the detection of newly synthesized immunoglobulins in lymphocyte cultures treated with PHA is the concomitant synthesis of the large amounts of protein associated with the cell growth and division involved in transformation.

The present studies were undertaken to determine whether the transformed or transforming lymphocytes actively synthesize immunoglobulin and have employed both immunofluorescence and radioactive amino acid incorporation combined with controlled immunological precipitation as a means of detecting small amounts of newly formed specific protein. Lymphocyte donors consisted of normal individuals and Hashimoto patients with high titre anti-thyroglobulin antibodies. Preliminary studies on tuberculin purified protein derivative (PPD) induced transformation of peripheral blood lymphocytes from Mantoux positive donors, and on pokeweed mitogen stimulated cells have also been included. Some of the results have been briefly described elsewhere (Greaves, 1966; Greaves & Roitt, 1967).

MATERIALS AND METHODS

Mitogens

PHA (Burroughs Wellcome) was pooled from a number of vials and used at an optimal

concentration of 100 μg/ml. Pokeweed was obtained as a gift from Dr J. G. Feinberg (Miles Laboratories, Stoke Poges) and the active principle purified from the extracts by the method of Börgeson *et al.* (1966); the optimal dose was 150 μg protein/ml. Tuberculin (PPD) was dialysed against normal saline and sterilized by filtration through Millipore membranes prior to use; the optimal concentration for transformation of lymphocytes from Mantoux positive donors was 100–250 units/ml. Staphylococcal filtrate was a gift from Dr S. Knight (Department of Experimental Pathology, Birmingham) and was used at a concentration giving optimal transformation. Anti-lymphocyte serum was prepared by intravenous injection of goats with human spleen and tonsil cells (Greaves *et al.*, 1967); the optimal concentration for stimulation of lymphocytes in the absence of complement was found to be 0·04 ml/ml culture medium.

Antisera

Specific antisera to IgG, IgA and IgM were obtained from the Central Laboratories for Blood Transfusion, Dutch Red Cross (Amsterdam). Rabbit anti-human thyroglobulin was obtained by immunizing animals with an ammonium sulphate precipitated preparation of thyroglobulin in Freund's adjuvant followed by intravenous boosting using the alum precipitated protein. The final antiserum was absorbed with human serum before use. Immunological specificity of all sera was established using immunoelectrophoresis. For the immunofluorescent technique the sera were precipitated with 18% sodium sulphate and the globulins conjugated with fluorescein isothiocyanate using the method of Marshall, Eveland & Smith (1958) and absorbed with acetone dried guinea-pig liver powder before use (50 mg/ml) except where stated. The fluorescein–protein ratio of all absorbed conjugates was between 0·7 and 1·0.

Cell culture

Venous blood, 20–60 ml, was taken either from normal donors or from Hashimoto patients and transferred to universal containers with 20 units of phenol-free heparin. A minimum of 10 ml blood was set aside in an unheparinized container to provide a supplement of autologous serum for the medium. Erythrocytes were sedimented with a half-volume of 6% Dextran (molecular weight 220,000) in saline for 40 min at 37°C. The leucocyte rich suspension was incubated at 37°C for 90 min in a horizontal medical flat to allow attachment to the glass of monocytes and polymorphonuclear leucocytes. The supernatant usually contained 70–85% of lymphocytes with a recovery of 75–85%. The lymphocyte suspension was spun at 150 *g* for 6 min, washed in medium 199, centrifuged at 125 *g* for 4 min and resuspended in 20% autologous serum in leucine-free Eagle's medium to which had been added 1 μC of L-[^{14}C]leucine (7·8 μg, 150 mC/m-mole; Radiochemical Centre, Amersham)/ml medium. PHA was added to give an optimal concentration for transformation, the total volume being adjusted to give a lymphocyte count of 10^6 lymphocytes/ml; 1-ml cultures were set up in McCartney bottles and gassed with 5% CO_2–oxygen. Incubation was carried out at 37°C without agitation from 0 to 120 hr. Only cultures showing good cell growth and survival as shown by 1% trypan blue dye exclusion were studied. The following controls were employed: lymphocyte cultures lacking PHA; cultures treated with 10^{-4} M-puromycin with and without PHA; cell free culture medium, with and without PHA. Transformation was assessed by examination of cell smears stained with May–Grünwald–Giemsa. In

experiments with mitogens other than PHA, the cultures were set up in 20% autologous serum in complete Eagle's medium without radioactive amino acids.

Investigation of proteins synthesized in cultures

A proportion of the cultures were used directly for immunofluorescence studies, the remaining cultures (cells plus medium) being extracted as indicated in Fig. 1. The cultures were subjected to three cycles of freezing at −20°C and thawing and then treated with ultrasound for 4–5 min at a nominal 50 W and 16–24 kcyles/sec using a Mullard instrument (Mullard Ltd, London; type E7680). Remaining particulate matter was spun down at 38,000 *g* for 1 hr at 4°C. The supernatant contained 75–85% of the incorporated label; one-half was dialysed against saline and used to determine trichloroacetic acid precipitable protein or for specific immunological precipitations. The other half was pooled with similar fractions from other cultures and studied by radioelectrophoresis and radioimmunoelectrophoresis after concentration and passage down a column of Sephadex G-25 to remove free label.

Total protein synthesis. Diluted extract, 0·2 ml, was added to an equal volume of cold 10% trichloroacetic acid (TCA), washed twice in cold 5% TCA and once in 5% TCA at 90°C for 15 min to remove unwanted radioactive compounds (Van Furth *et al.*, 1966), before resuspension in formic acid and transfer to pre-weighed aluminium planchettes. The precipitates, consisting of 4–8 mg of protein, were counted at infinite thinness (self absorption <5%) using a Nuclear Chicago gas flow counter. The coefficient of variation of the counts was <6%.

Specific immunological precipitation. Before precipitation with specific antibody, non-specific complex formation was carried out *in situ* as described by Stravitsky (1958) in order to give a measure of adventitious co-precipitation of labelled material in this system. Complexes were formed in the diluted extract by the sequential addition of ovalbumin and rabbit anti-ovalbumin serum at optimal proportions to give a precipitate of approximately the same weight as that obtained in the final precipitation of immunoglobulin. After incubation at 37°C for 30 min and overnight at 4°C, the complexes were spun down and discarded. The supernatant was subjected to a second treatment with ovalbumin to give a control precipitate. Specific complexes were formed by adding to this supernatant either a specific anti-IgG or anti-IgM or purified human thyroglobulin. These precipitates and the second non-specific precipitate were washed three times in cold phosphate buffered saline (PBS) and once in 5% TCA at 90°C before measurement of the radioactivity as described above.

Radioelectrophoresis. Approximately 2–4-μl samples of concentrated culture extract were applied centrally to duplicate strips of cellulose acetate for electrophoresis in veronal buffer (pH 8·5; $I = 0{\cdot}1$) a current of 1 mA/strip being applied for 2 hr. One of the strips was stained with 0·002% nigrosin in 2% acetic acid and scanned for protein; the other was dried, mounted on backing paper and scanned for radioactivity, using a Packard Radiochromatogram scanner.

Radioimmunoelectrophoresis. Samples of concentrated culture extract were subjected to micro-immunoelectrophoresis in agar (Scheidegger, 1955) and developed with a broad spectrum anti-immunoglobulin serum and with specific anti-IgG and anti-IgM. The plate was washed extensively with normal saline and then water; after drying they were exposed to Kodak X-ray film for 6–12 weeks.

Immunofluorescence. Preparations of cells for immunofluorescent staining were made by the method of Balfour *et al.* (1965). Cells were washed twice in PBS, once in PBS containing 5% bovine serum albumin, resuspended in PBS and deposited on glass slides as flattened preparations with clearly defined cytoplasm by spinning at 450 rev/min for 15 min in a centrifugal apparatus (Doré & Balfour, 1965). The cells were fixed in 95% ethanol for 7 min, washed in 0·01 M-barbitone buffer, pH 7·2, containing 0·85% sodium chloride and stained with the appropriate anti-immunoglobulin conjugate in a humid chamber for 25 min. The smears were then washed for 15 min in barbitone buffer and mounted in 1:1 glycerol–barbitone buffer, pH 8·6. The appropriate dilutions of conjugate were assessed by prior staining of human tonsil lymphoid cells under similar conditions. Controls included pretreatment with unconjugated specific antisera and use of conjugates of anti-human β_{1C}, anti-fibrinogen, anti-ovalbumin and bovine serum albumin having similar protein–fluorochrome molar ratios and protein content as the specific conjugates. In some instances, cells were stained for anti-thyroglobulin by the sandwich technique; fixed smears were treated with human thyroglobulin (10 mg/ml) for 30 min prior to addition of the rabbit anti-thyroglobulin conjugate. Cells cultured from Mantoux positive donors in the presence of specific antigen were stained with fluorescein conjugates of PPD. At least 1000 cells on duplicate slides were counted to determine the percentage of positively stained cells.

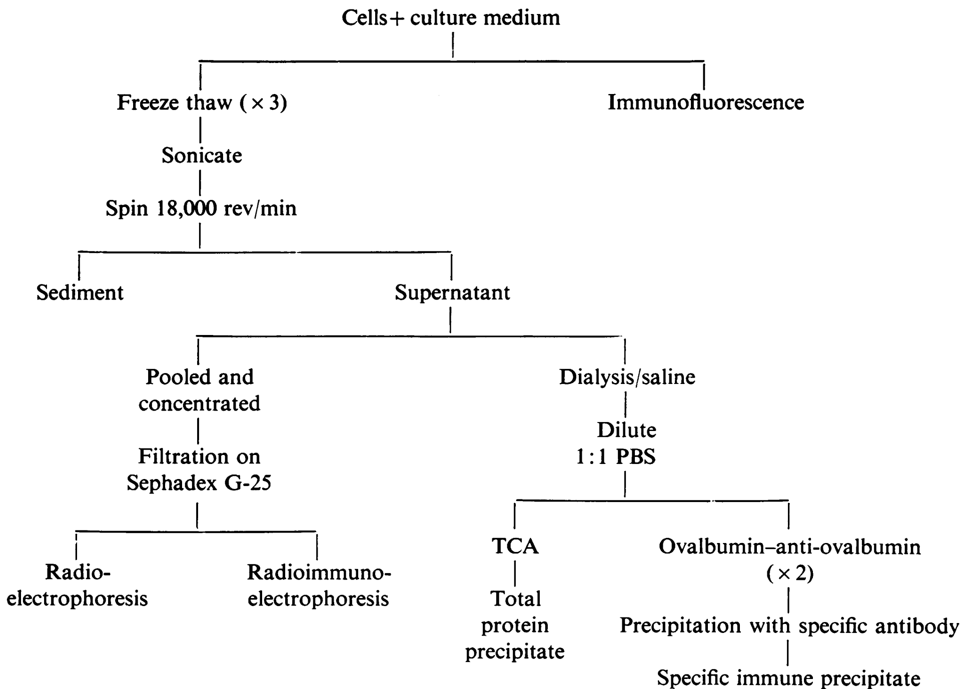

FIG. 1. Investigation of proteins synthesized in lymphocyte cultures.

In certain experiments the immunofluorescent staining method of Ripps & Hirschhorn (1967) using live cells after 72 hr in culture, was followed. The cells were washed three times in Eagle's minimal essential medium, resuspended in the latter and incubated with different

amounts of fluorescent conjugate for 45 min at 37°C in a shaking water-bath; cells were then washed three times in Eagle's medium, prepared as smears by centrifugation and mounted in 1:1 glycerol–barbitone buffer.

Preparations were observed with a Reichert Zetopan ultraviolet microscope fitted with a Tiyoda darkground condenser (barrier filter, UGl/1·5 mm; absorption filter, GG9/1·0 mm). Photographs were taken with high speed Ektachrome, ASA 160, using exposure times of 2–4 min.

RESULTS

Effect of phytohaemagglutinin on protein synthesis

Lymphocyte cultures were incubated in the presence and absence of phytohaemagglutinin using the incorporation of [^{14}C]leucine for the assessment of protein synthesis as described under 'Materials and methods'. Cell extracts were studied by trichloroacetic acid precipitation of total protein, by specific immunological precipitations and by radioelectrophoretic and radioimmunoelectrophoretic analyses as shown in Fig. 1.

Total protein synthesis. Both normal and Hashimoto peripheral blood lymphocytes gave typical transformation after 24–72 hr of culture with PHA (Table 1).

TABLE 1. Transformation of lymphocytes by phytohaemagglutinin

Donors	No. of donors	% of cells surviving at 72 hr*	% blast cells/ surviving cells at 72 hr
Normal	16	72·1 ± 11·4	83·2 ± 8·0
Hashimoto	22	70·8 ± 15·4	81·2 ± 11·6

* Mean ± S.D.

As indicated in Fig. 2 protein synthesis was considerably enhanced in cultures to which PHA was added. Differences between PHA and untreated cultures were apparent within a few hours. Practically no label was incorporated into protein in the absence of cells and furthermore puromycin, an inhibitor of protein synthesis (Campbell, 1965) gave a graded inhibition of incorporation over a molarity range of 10^{-3}–10^{-7}. Preliminary experiments also established that after 72 hr of culture over 90% of protein synthesized in PHA cultures was retained intracellularly.

Immunological precipitation of immunoglobulins. As indicated in Fig. 3 non-specific co-precipitation of label with ovalbumin–anti-ovalbumin complexes increased throughout the culture period and was considerably greater in PHA treated cultures than in untreated controls. Very little co-precipitation of label occurred in controls containing 10^{-4} M-puromycin or in culture medium devoid of cells. Three donors, two Hashimoto and one normal, were investigated for synthesis of immunoglobulins using specific precipitation. In extracts from 72-hr cultures, there was no evidence for synthesis of IgM or anti-thyroglobulin antibody in the presence or absence of PHA and only in the case of donor B.T. was the radioactivity of the specific IgG complexes greater than that of the preceding non-specific

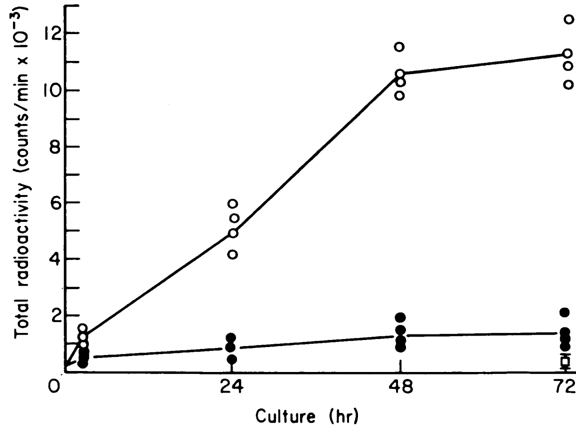

FIG. 2. Effect of phytohaemagglutinin (PHA) on total protein synthesis in lymphocyte cultures assessed by incorporation of [^{14}C]leucine. ○, PHA-treated; ●, untreated; □, controls.

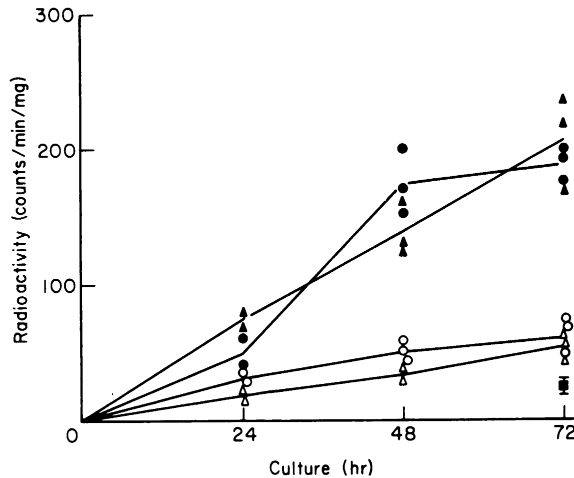

FIG. 3. Non-specific co-precipitation of [^{14}C]leucine by ovalbumin–anti-ovalbumin complexes in extracts of PHA-treated (▲, Hashimoto donor; ●, normal donor) and untreated (△, Hashimoto donor; ○, normal donor) cultures. Controls (■) consisted of cultures containing 10^{-4} M-puromycin or of culture medium devoid of cells both with or without PHA. The mean value ±S.D. is shown.

precipitate (Table 2). This IgG synthesis occurred during the first 24 hr and was only clearly evident in phytohaemagglutinin stimulated cultures (Fig. 4); after this time the rate of incorporation of radioactivity paralleled that found in the non-specific precipitates.

Radioelectrophoresis. After electrophoresis of concentrated culture extracts on cellulose acetate, there was an uneven but continuous distribution of radioactivity over the α- and

TABLE 2. Immunological precipitation of synthesized immunoglobulins (72-hr culture extracts)

Lymphocyte donor (status)	PHA added	Radioactivity of immune complexes (counts/min/mg)			
		Second non-specific precipitation ovalbumin–anti-ovalbumin	Subsequent specific immunological precipitations		
			Anti-IgG	Anti-IgM	Human thyroglobulin
B.T.	+	206·7	274·3	190·0	99·3
Hashimoto	–	54·0	79·0	42·7	32·8
F.E.	+	188·5	174·4	131·8	123·0
Hashimoto	–	60·1	43·4	27·0	20·0
J.C.	+	220·0	211·8	170·0	
Normal	–	96·1	92·3	66·6	

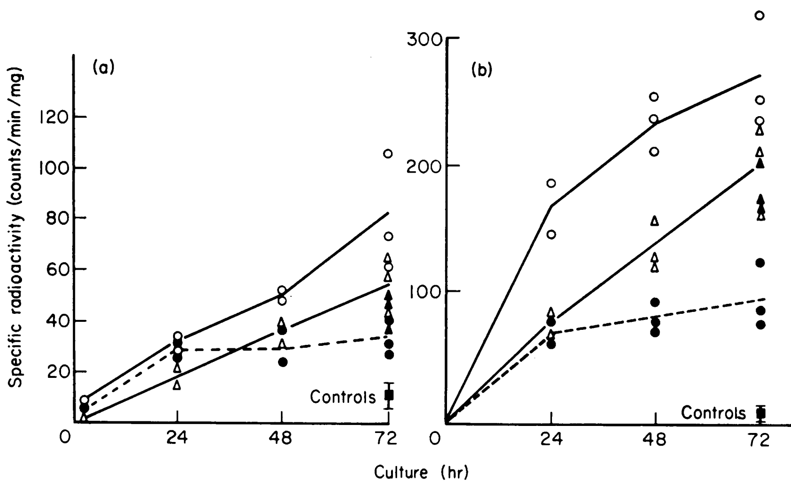

FIG. 4. Time course of incorporation of [^{14}C]leucine into immunoglobulins in lymphocyte cultures in the absence (a) and presence (b) of PHA. Lymphocytes were from Hashimoto donor B.T. Controls (■) as in Fig. 3. Precipitate formed by: ○, anti-IgG; △, ovalbumin–anti-ovalbumin; ▲, anti-IgM; ●, thyroglobulin.

β-globulin regions with a small peak at the origin indicative of degraded material (Fig. 5). Considerably more label was present in PHA extracts as compared with untreated cultures. Only a very small amount of radioactivity was detected in the γ-globulin region. The significance of the marginal increase in the radioactivity of proteins with γ-mobility in PHA treated cultures was difficult to assess.

Radioimmunoelectrophoresis. Concentrated extracts of 72-hr cultures were subjected to immunoelectrophoresis against anti-human serum and anti-human immunoglobulin; the slides were washed and exposed to X-ray film for 6–12 weeks. Radioactivity was distributed around the central well and along a central region with α–β-globulin mobility, considerably

more being present in the PHA-stimulated cultures. The only precipitin arcs consistently labelled in PHA and untreated cell extracts corresponded to an α_2-macroglobulin and an α_1-lipoprotein; in the latter, radioisotope was restricted to the cathodic region. Exposure of plates for 12 weeks revealed faint labelling of the IgG line in extracts of lymphocyte cultures from both a Hashimoto and a normal donor, although no quantitative differences between PHA and untreated culture extracts were evident. No other immunoglobulin arcs were detectably labelled.

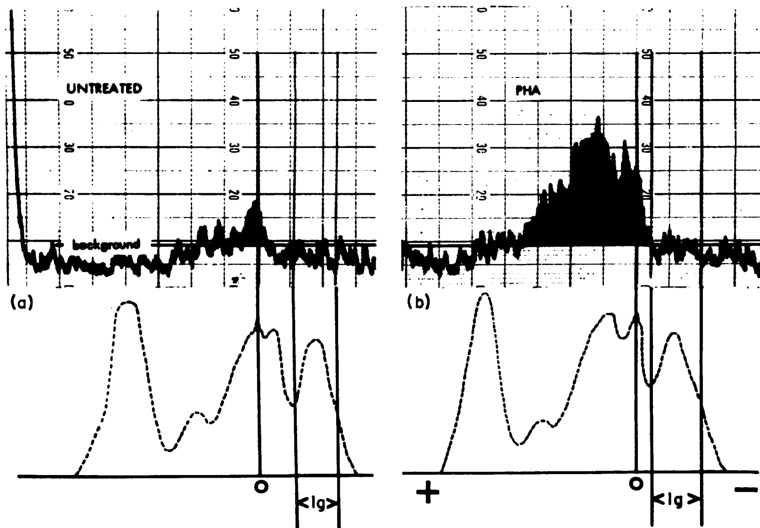

FIG. 5. Radioelectrophoresis of cell extracts obtained after culturing lymphocytes for 72 hr with [^{14}C]leucine in the absence (a) and presence (b) of PHA. Top, Radioactivity scan of cellulose acetate strip; bottom, protein scan of stained strip; o, origin; Ig, γ-globulin peak.

Immunofluorescent studies on mitogen stimulated lymphocytes

Staining of fixed cells. The results of immunofluorescent investigations on fixed smears of human lymphocytes from cultures treated with different mitogens are presented in Table 3. A study of peripheral blood lymphocyte suspensions prior to culture revealed a very small percentage of cells, mostly medium sized lymphocytes, staining for IgG and less frequently IgM. Two per cent of lymphocytes from donor B.T. contained detectable IgG compared with values of 0·7% and 0·9% for donors F.E. and J.C. No cells positive for IgA were observed. A very small percentage of cells staining for anti-thyroglobulin antibody by the sandwich technique were occasionally but not consistently observed in suspensions of peripheral blood lymphocytes from Hashimoto patients but not in cells derived from normal individuals. Lymphocytes from both normal and Hashimoto donors were cultured with PHA and harvested at 2, 4, 8, 24, 48, 72 and 120 hr. During the early period of culture, the number of cells giving positive staining declined from the initial value and indeed after 24 hr, fluorescent labelling of cells was only observed rarely in either PHA-treated or untreated cultures. Blast cells from PHA cultures were uniformly negative for IgG, IgM, IgA

TABLE 3. Immunofluorescent staining for immunoglobulins in fixed preparations of human peripheral blood lymphocytes

Cell donor	No. of experiments	Hours in culture	Added mitogen	% of cells staining with conjugates of:							
				Anti-Ig	Anti-IgG	Anti-IgM	Anti-IgA	Tg	Anti-Tg (Tg sandwich)	PPD	Control conjugates
Normal	4	0	—	0·1–1·5	0·1–1·5	0·1–0·2	—	—	—		—
	6	2–24	—	0·1–0·5	0·1–0·5	—	—	—			—
	6	24–120	—	—	—	—	—	—			—
	6	2–24	PHA	0·1–0·5	0·1–0·5	—	—	—			—
	6	24–120	PHA	—	—	—	—	—	—		—
Hashimoto	5	0	—	0·1–2·0	0·1–2·0	0·1–0·3	—	0·1–0·2	0·1–0·2		—
	5	2–24	—	0·1–0·5	0·1–0·5	—	—	—	—		—
	5	24–120	—	—	—	—	—	—	—		—
	5	2–24	PHA	0·1–0·5	0·1–0·5	—	—	—	—		—
	5	24–120	PHA	—	—	—	—	—	—		—
Normal	2	72	*Staph.* filtrate	—	—						—
Normal	3	72	ALS	—	—						—
Normal	4	48–120	Pokeweed	1–4·0	1–4·0	0·1–1·0	0·1–0·4	—			—
Hashimoto	1	48–120	Pokeweed	3·0	2·5	0·25	—	—	—		—
Normal (Mantoux + ve)	4	120	PPD	1–2·0						1–2·0	

— = < 0·1%; blank = not done.

and anti-thyroglobulin antibody (Fig. 6b). Blast cells in smears of lymphocytes from cultures treated with anti-lymphocyte serum (Fig. 6d) or staphylococcal filtrate were also unstained by anti-immunoglobulin conjugates.

Positive staining for immunoglobulin was, however, observed in a small percentage of the blast cells obtained in PPD stimulated cultures derived from Mantoux positive donors

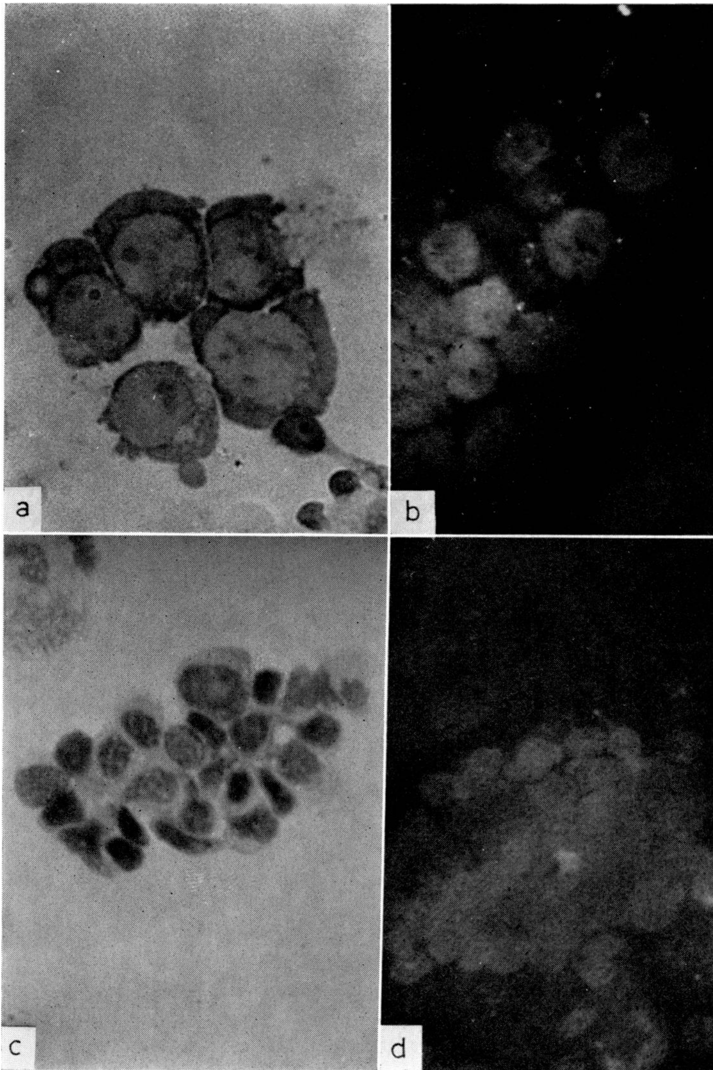

FIG. 6. (a) PHA-stimulated blast cells stained with May–Grünwald–Giemsa. (b) PHA-stimulated lymphocytes after 72-hr culture, fixed and treated with anti-human immunoglobulin conjugate. Negative reaction. (c) ALS (anti-lymphocyte serum)-stimulated cells after 72-hr culture stained with May–Grünwald–Giemsa. (d) ALS-stimulated cells after 72-hr culture, fixed and treated with anti-human immunoglobulin conjugate. Negative reaction.

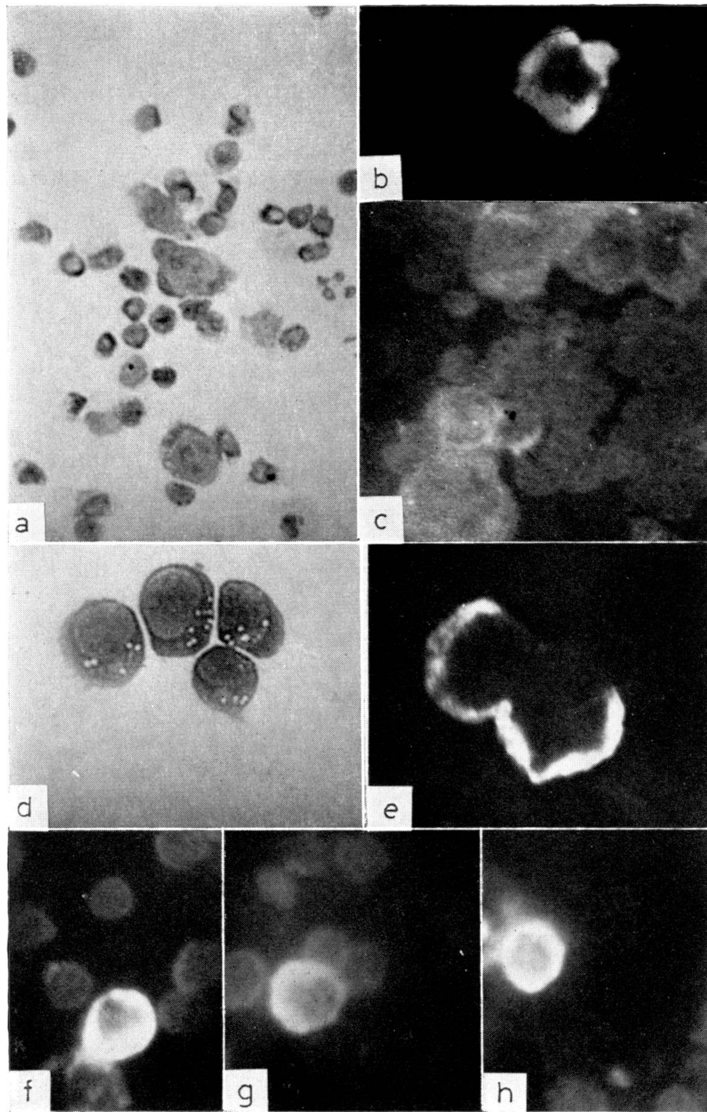

FIG. 7. (a) PPD-stimulated cells from a Mantoux positive donor after 5 days in culture stained with May–Grünwald–Giemsa. (b) PPD-stimulated cell from above culture fixed and stained with anti-human immunoglobulin. Positive reaction. (c) Same preparation as (b) showing a group of unstained cells. (d) Burkitt lymphoma cells, EB2 line, stained with May–Grünwald–Giemsa. (e) EB2 Burkitt lymphoma cells, fixed and stained with anti-human IgG conjugate. Positive reaction. (f) Fixed human tonsil cells stained with anti-IgG conjugate. (g) Fixed human tonsil cells stained with anti-IgA conjugate. (h) Fixed human tonsil cells stained with anti-IgM.

(Fig. 7b); the majority were, however, negative (Fig. 7c). An appreciable number of fluorescent cells were seen after treatment of the transformed lymphocytes with a PPD conjugate. No fluorescent reactions were obtained with the blast cells in PHA-treated cultures derived from the same donor.

One to four per cent of cells from pokeweed stimulated cultures consistently stained for

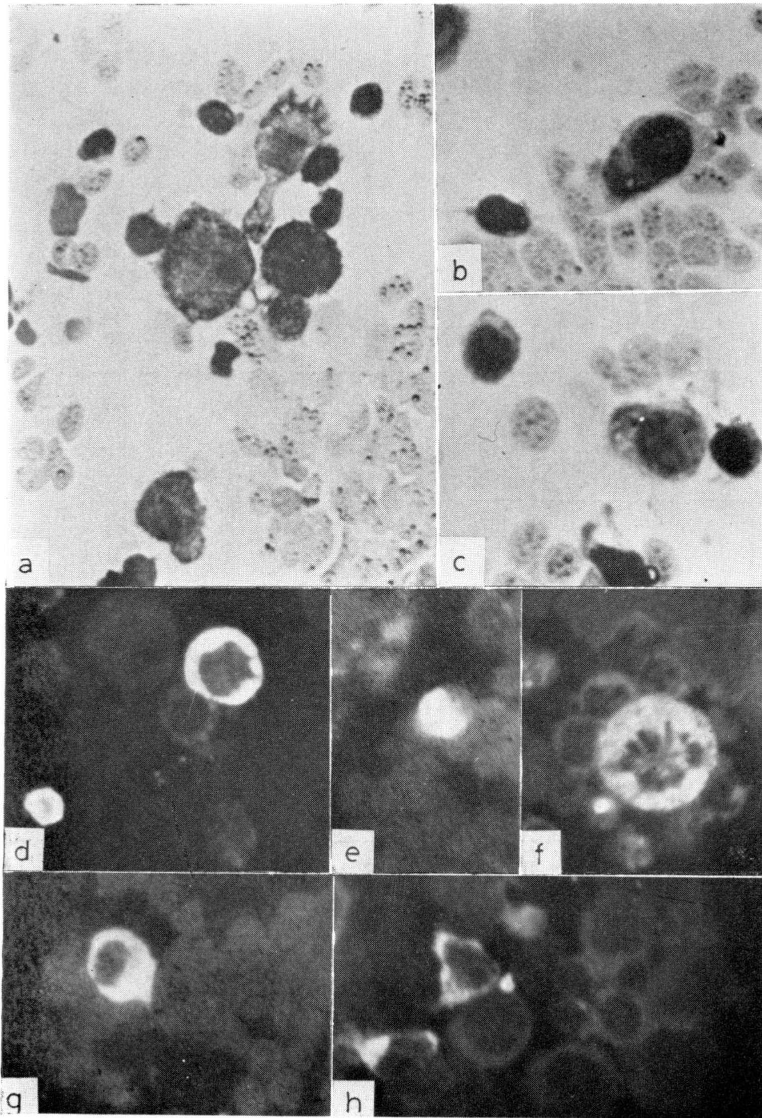

FIG. 8. (a) Pokeweed-stimulated lymphocytes after 72-hr culture stained with May–Grünwald–Giemsa showing blast forms similar to those in PHA cultures. (b, c) Same preparation as (a) showing cells not observed in PHA cultures. (d, e, f) Pokeweed-stimulated cells after 72-hr culture, fixed and stained with fluorescent anti-immunoglobulin. (g, h) As above stained with anti-IgG conjugate.

immunoglobulin and in particular for IgG (Fig. 8d–h). Pokeweed stimulated cells from a Hashimoto donor did not stain for anti-thyroglobulin antibody.

Of five lines of Burkitt lymphoma cells investigated, lines EB2 and EB3 showed a small percentage of cells (2–6%) positively stained for IgG (Fig. 7e). These cells are morphologically similar to PHA blasts (Pulvertaft, 1964) and line EB2 has been shown to synthesize IgG *in vitro* (Fahey *et al.*, 1966). In order to provide further information on the reliability of the techniques and reagents used to demonstrate intracellular immunoglobulins, preparations made from human tonsils were studied; 1–4% of the cells stained for IgG (Fig. 7f), 0·1–1% for IgA (Fig. 7g) and 0·1–1% for IgM (Fig. 7h) in accord with previous reports (Crabbé & Heremans, 1967).

Staining of viable cells. The results with Ripps and Hirschhorn's technique involving addition of fluorescent conjugate to suspensions of viable cells are shown in Table 4. In marked contrast to the results with fixed cells, over half the PHA stimulated cells appeared

TABLE 4. Immunofluorescent staining of suspensions of viable human lymphocytes

Conjugate	Liver powder absorption 50 mg/ml	FITC/protein molar ratio	mg protein/ml	Volume added/ml cell suspension	% cells staining	
					Untreated	PHA-treated
Rabbit anti-human immunoglobulin (γ-globulin fraction)	–	2·0	11·0	0·01	0·5	5·5
				0·025	0·5	15·5
				0·05	2·0	45·0
				0·10	3·0	52·5
	+	0·8	11·0	0·01	0·5	0·5
				0·025	0·5	4·5
				0·05	0·5	9·5
				0·10	1·0	14·0
Rabbit anti-ovalbumin (γ-globulin fraction)	–	2·1	12·5	0·01 to 0·05	0·5	5·0

Normal human peripheral blood lymphocytes cultured for 72 hr in the presence and absence of PHA in 20% foetal calf serum were treated directly with conjugate. Damaged cells which often stained brightly were disregarded.

to take up the anti-immunoglobulin conjugate. However, it was necessary to use an unabsorbed conjugate to obtain convincing staining. Up to 50% of the cells were fluorescent with a wide range of staining intensity (Fig. 9a). Absorption with liver powder lowered the fluorescein to protein molar ratio of the conjugate from 2·0 to 0·8 and considerably reduced its ability to give positive reactions. The unabsorbed rabbit anti-ovalbumin conjugate used as a control stained no unstimulated cells but gave positive reactions with a small number of blast cells. This non-specific uptake could be greatly enhanced by simultaneous addition of PHA or anti-lymphocyte serum (Fig. 9b) and to a lesser extent by unconjugated anti-IgG.

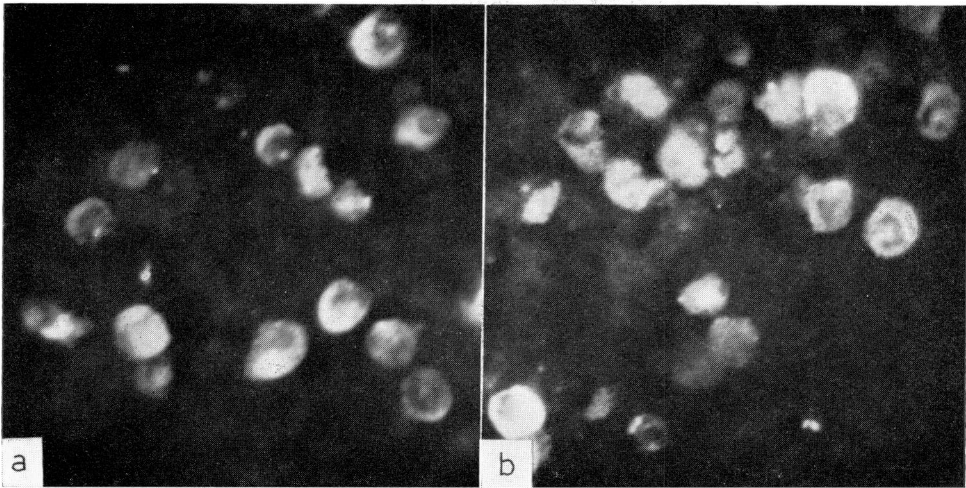

FIG. 9. (a) PHA cells from 72-hr culture unfixed and treated in suspension with conjugated anti-IgG. Positive reaction in many cells. Compare negative reactions with fixed cells, Fig. 6 (b). (b) Same culture as above, unfixed and treated in suspension with a mixture of ALS and fluorescein-conjugated rabbit-anti-ovalbumin showing a similar reaction.

DISCUSSION

PHA considerably enhanced protein synthesis in peripheral blood lymphocytes under the conditions of culture employed in the present investigations. The lack of incorporation of isotopic amino acid in the puromycin and cell-free culture extracts suggests that the increase in incorporation of label with PHA was indicative of true *de novo* protein synthesis. The effect was detectable within a few hours of the addition of PHA and levelled off during the 3rd day of culture, by which time the majority of the lymphocytes had fully transformed into blast cells. Presumably much of the protein synthesized is associated with the cellular events of growth and division as has been observed in a number of mammalian tissues maintained *in vitro* (Bullough, 1965). A number of different attempts were made to assess the extent to which PHA was also capable of stimulating immunoglobulin synthesis. The radioimmunoelectrophoretic experiments suggested that at the most only a very small amount of immunoglobulin was synthesized by peripheral blood lymphocytes in this culture system and differences between the amounts recovered from PHA and untreated cell cultures were not demonstrable. This accords with the experience of Sell *et al.* (1965), Huber *et al.* (1967) and of Epstein (1966). Some labelling of the IgG arc was visible in the immunoelectrophoretic autoradiographs published by Turner & Forbes (1966) but assessment of the specificity of labelling in this technique is difficult; thus radioactivity in the α_1-lipoprotein and α_2-macroglobulin arcs, also observed in our own experiments, has been ascribed to non-specific co-precipitation rather than *de novo* synthesis (Thorbecke, Hochwald & Jacobson, 1962; Epstein, 1966). Radioelectrophoresis in the present study and in those of others (Huber *et al.*, 1967; Turner & Forbes, 1966) indicated trace amounts of radioactivity in the γ-globulin region in extracts from PHA-stimulated cells; this may indicate synthesis of some γ-globulin

although it is difficult to exclude the possibility that other labelled proteins may have a similar electrophoretic mobility (Budzynski *et al.*, 1962).

The identification of newly synthesized radioactive proteins by precipitation with specific antisera is a technique commonly employed yet gives results which can be significantly affected by the non-specific co-precipitation of radioactive materials. The absence of this non-specific effect in dialysed extracts from puromycin treated cells suggests that the phenomenon is due to co-precipitation of other newly synthesized proteins or intermediates which tend to bind to the immune complexes formed *in situ*. Thus it is generally recommended (Hochwald, Thorbecke & Asofsky, 1961) that two successive independent immune precipitations, e.g. ovalbumin–anti-ovalbumin, are carried out *in situ* before addition of specific antibody to the supernatant. The counts in this precipitate should be substantially above that of the second non-specific immune complex if the results are to be evaluated with any confidence (Stavitsky, 1961). Since PHA stimulates the general synthesis of cellular proteins in lymphocyte cultures, this must proportionately increase the non-specific radioactivity precipitated as could be clearly seen in the present experiments; failure to control this non-specific effect adequately would lead to an overestimate of the amount of immunoglobulin synthesized by PHA cultures as judged by the counts in the final specific immune precipitate (Forbes, 1965; Parenti *et al.*, 1966; Ripps & Hirschhorn, 1967). In our studies specific immunoglobulin synthesis was detected in the lymphocyte cultures from only one out of three donors; this was IgG and occurred during the first 24 hr *in vitro*. In the presence of PHA this synthesis appeared to be marginally enhanced.

Immunofluorescent staining of alcohol-fixed smears of peripheral blood lymphocytes showed a small proportion of cells staining for immunoglobulin (cf. Van Furth *et al.*, 1966) but after a short period in culture this number decreased so that by 24 hr none was detectable. It seems likely that these immunoglobulin containing cells were responsible for the small amount of IgG synthesized in cultures of donor B.T.'s lymphocytes during the first 24 hr. The slight enhancement of synthesis by PHA during the first 24 hr in our studies with donor B.T.'s lymphocytes may have been due to an effect on the short-term survival of these immunoglobulin synthesizing cells or perhaps to an overall stimulation of their metabolism. It is unlikely that the immunoglobulin synthesis could be ascribed to blast cells since these were unequivocally negative in the immunofluorescent tests. Negative results were also obtained with blast cells stimulated by anti-lymphocyte serum and staphylococcal filtrate. With the same fluorescent reagents and with identical techniques, however, it was possible to detect immunoglobulin in a small percentage of the blasts in PPD-transformed cultures from Mantoux positive donors and in a slightly higher proportion of cells in pokeweed mitogen-treated cultures; the numbers corresponded approximately with the proportion known to have a well-developed rough-surfaced endoplasmic reticulum (Douglas *et al.*, 1967b), although this itself may not be an obligatory criterion for considering a lymphoid cell to be an active producer of antibody (Harris, Hummeler & Harris, 1966; Douglas, Borjeson & Chessin, 1967a). A similar percentage of PPD stimulated cells also appeared to react with conjugated PPD. The bright staining of tonsil preparations and of Burkitt lymphoma cell lines EB_2 and EB_3 further confirms the adequacy of the techniques employed for the demonstration of intracellular immunoglobulin, accepting the limitation imposed by sensitivity.

While similar findings with PHA-stimulated blasts have been described by others (Ling *et al.*, 1965; Sell *et al.*, 1965; Balfour *et al.*, 1965; Huber *et al.*, 1967), Ripps & Hirschhorn

(1967) have reported that the majority of these cells and those stimulated by specific antigen can be stained for all three major immunoglobulin classes. The discrepancy in results probably lies in their different staining procedure which involves incubation of the viable cells with fluorescent conjugate at 37°C. Using the same technique we have also been able to demonstrate intracellular staining of a considerable percentage of blast cells. To obtain convincing results it was, however, necessary to use an unabsorbed conjugate with a relatively high fluorescein–protein molar ratio in the same way that hyperconjugated antisera have been employed for the demonstration of cell membrane histocompatibility antigens with suspension staining techniques (Moller, 1961; Cerottini & Brunner, 1967).

Antibodies do not normally penetrate undamaged cells except by endocytosis (Hiramoto, Goldstein & Pressman, 1960). Since this process can be stimulated by the combination of antibody with antigen on the cell surface as shown with ascites tumour cells (Easton, Goldberg & Green, 1962) and macrophages (Cohn & Parks, 1967), the positive results obtained with the suspension staining technique could be accounted for if one postulated the existence of immunoglobulin molecules associated with the surface membrane of the PHA blast which function as cell markers. The specificity of the reaction would depend initially on the stimulation of micro-pinocytosis by combination of the conjugate with the surface marker giving rise to the secondary intracellular accumulation of labelled protein from the medium on a non-immunological basis; indeed our own preliminary studies have demonstrated a non-specific uptake of irrelevant fluorescein conjugate by PHA-blasts treated with PHA, anti-lymphocyte and anti-immunoglobulin sera under similar conditions particularly when the heavily conjugated protein molecules are not first removed by absorption with liver powder. Thus, in Hirschhorn's experiments blast cells derived from agammaglobulinaemic donors would not 'stain' because they did not possess the appropriate immunoglobulin markers whereas the donor with IgA deficiency failed to 'stain' with anti-IgA because of a defect of specific IgA marker. The inhibition of conjugate uptake by prior incubation of the cells with unconjugated antibody could be due to blocking of the markers associated with a transient endocytosis in the first reaction preventing combination with labelled antibody on subsequent addition. The low incidence of staining with unstimulated cells relative to PHA blasts using this technique may be due to the increased metabolic activity of the blast cells with greater energy potential for endocytosis, or to a change in the configuration of the cell membrane on transformation which allows preformed markers to become more accessible, or perhaps to the replication of immunoglobulin markers during blast-cell formation. There is increasing evidence for the presence of immunoglobulin markers on the lymphocyte surface. Although not present in sufficient concentrations to be detected by immunofluorescence of fixed cell preparations, such markers would appear to be implicated in the transformation of rabbit blood lymphocytes by anti-allotypic and anti-immunoglobulin sera (Sell & Gell, 1965; Sell, 1967). Furthermore, the production of electrophoretically fast antibody in response to a basic antigen and of slower migrating antibody on immunization with more acidic antigens (Robbins *et al.*, 1967) is most readily understood on the basis of selection of cell lines with preformed antibody of opposite charge on their surface preferentially binding and thence being stimulated by antigen. Unlike the rabbit system, antisera to human immunoglobulins are not particularly effective in stimulating transformation of human lymphocytes (Holt, Ling & Stanworth, 1966; Greaves, unpublished observations) although Oppenheim, Rogentine & Terry (1967) have reported a small response to monkey antiserum. The poor response of human peripheral blood

lymphocytes to anti-immunoglobulin sera relative to rabbit lymphocytes is perhaps more readily interpreted as a quantitative deficiency of suitably positioned immunoglobulin molecules rather than a total lack of these on or in the human small lymphocyte. Assuming such membrane bound immunoglobulin molecules do exist, it is conceivable that during transformation induced by both non-specific mitogens and by antigens, some replication of these markers may take place, although it is unlikely that this would be of sufficient magnitude to be detectable by amino-acid incorporation techniques.

If replication of immunoglobulin markers does occur during PHA stimulation, then in a limited sense these blast cells could be said to be synthesizing immunoglobulin but there is no question of their producing conventional humoral antibody or intracytoplasmic accumulation of immunoglobulin. Thus PHA did not increase anti-thyroglobulin antibody in cultures from Hashimoto donors, nor did it increase the number of plaque-forming cells in lymphocyte cultures from rabbits primed with sheep red blood cells even though excellent secondary responses were obtained *in vitro* with specific antigen (Harris & Littleton, 1966; Greaves & Flad, to be published). Cells capable of transformation by PHA may be more closely related to cell-mediated immunity in so far as the mitogenic response to PHA is greatly diminished in pathological states associated with impaired delayed hypersensitivity and in thymic deficiency in man (Lischner, Punnett & DiGeorge, 1967), the rat (Meuwissen *et al.*, 1967) and the chicken (Greaves, Rose & Roitt, to be published). The synthesis of immunoglobulin surface markers could be related to the production of a 'cell-bound antibody' often postulated to provide the basis for immunological specificity in delayed-type hypersensitivity reactions. The majority of blast cells in antigen dependent transformation closely resemble those stimulated by PHA and presumably reflect the activation of lymphocytes implicated in cell-mediated immunity (Mills, 1966) whereas the minority showing well-developed endoplasmic reticulum and staining with anti-immunoglobulin conjugates may be derived from a small number of primed cells belonging to a separate population of lymphocytes (? of bursa-equivalent origin) capable of expressing humoral immunocompetence but insensitive to PHA.

ACKNOWLEDGMENTS

We wish to thank Dr M. A. Epstein for cultures of Burkitt lymphoma cells and Dr J. G. Feinberg for a gift of pokeweed. We thank Mrs G. Stead for help in preparation of the manuscript and Mr H. S. Drury, medical artist, for the diagrams. The work was supported by grants from the Medical Research Council, the Nuffield Foundation, the Arthritis and Rheumatism Council and the World Health Organization.

REFERENCES

Balfour, B.M., Cooper, E.H. & Alpen, E.L. (1965) Morphological and kinetic studies on antibody-producing cells in rat lymph nodes. *Immunology*, **8**, 230.

Börgeson, J., Reisfield, R., Chessin, L.N., Welsh, P. & Douglas, S.D. (1966) Studies on human peripheral blood lymphocytes *in vitro*. I. Biological and physicochemical properties of the Pokeweed mitogen. *J. exp. Med.* **124**, 859.

Bullough, W.S. (1965) Mitotic and functional homeostasis. A speculative review. *Cancer Res.* **25**, 1683.

Budzynski, A.Z., Broda, E., Kellner, G. & Frimmel, J.S. (1962) An examination of HeLa cancer cells for production of gamma-globulin. *Nature (Lond.)*, **196**, 892.

Campbell, P.N. (1965) The biosynthesis of proteins. *Prog. Biophys. molec. Biol.* **15**, 1.

CERROTINI, J.C. & BRUNNER, K.T. (1967) Localization of mouse isoantigens of the cell surface as revealed by immunofluorescence. *Immunology*, **13**, 395.

COOPERBAND, W.R., ROSEN, F.S., KIBRICK, S. & JANEWAY, C.A. (1966) Gamma-globulin synthesis by lymphocytes from normal and agammaglobulinaemic individuals. *J. clin. Invest.* **45**, 998.

COHN, Z.A. & PARKS, E. (1967) The regulation of pinocytosis in mouse macrophages. IV. The immunological induction of pinocytic vesicles, secondary lysosomes, and hydrolytic enzymes. *J. exp. Med.* **125**, 1091.

CRABBÉ, P.A. & HEREMANS, J.F. (1967) Distribution in human nasopharyngeal tonsils of plasma cells containing different types of immunoglobulin polypeptide chains. *J. lab. Invest.* **16**, 112.

DORÉ, C.F. & BALFOUR, B.M. (1965) A device for preparing cell spreads. *Immunology*, **9**, 403.

DOUGLAS, S.D., BORJESON, J. & CHESSIN, L.N. (1967a) Studies on human lymphocytes *in vitro*. IV. Comparative fine structural features of the established Burkitt lymphoma cell lines AL-1, EB-2 and phytomitogen transformed lymphocytes. *J. Immunol.* **99**, 340.

DOUGLAS, S.D., HOFFMAN, P.F., BORJESON, J. & CHESSIN, L.N. (1967b) Studies on human peripheral blood lymphocytes *in vitro*. III. Fine structural features of lymphocyte transformation by pokeweed mitogen. *J. Immunol.* **98**, 17.

EASTON, J.M., GOLDBERG, B. & GREEN, H. (1962) Demonstration of surface antigens and pinocytosis in mammalian cells with ferritin-antibody conjugates. *J. Cell Biol.* **12**, 437.

ELVES, M.W., ROATH, S., TAYLOR, G. & ISRAELS, M.C.G. (1963) The *in vitro* production of antibody lymphocytes. *Lancet*, **i**, 1292.

EPSTEIN, L.B. (1966) Protein synthesis in phytohaemagglutinin stimulated cultures of human lymphocytes. *Fed. Proc.* **25**, 439.

FAHEY, J.L., FINEGOLD, I., RABSON, A.S. & MANAKER, R.A. (1966) Immunoglobulin synthesis *in vitro* by established human cell lines. *Science (N.Y.)*, **152**, 1259.

FORBES, I.J. (1965) Specific and non-specific stimulation of antibody synthesis by human leucocytes *in vitro*. *Lancet*, **i**, 198.

GREAVES, M.F. (1966) The effect of PHA on Ig synthesis in cultures of normal and Hashimoto patients' peripheral blood lymphocytes. Proceedings of the Symposium *The Biological Effects of Phytohaemagglutinin* (Ed. by M. W. Elves), p. 53. The Robert Jones and Agnes Hunt Orthopaedic Hospital Management Committee, Oswestry.

GREAVES, M.F. & ROITT, I.M. (1967) Blasts in lymphocyte cultures. *Lancet*, **ii**, 419.

GREAVES, M.F., ROITT, I.M., ZAMIR, R. & CARNAGHAN, R.B.A. (1967) Effect of anti-lymphocyte serum on responses of human peripheral blood lymphocytes to specific and non-specific stimulants *in vitro*. *Lancet*, **ii**, 1317.

HARRIS, T.N., HUMMELER, K. & HARRIS, S. (1966) Electron microscope observations on antibody producing lymph node cells. *J. exp. Med.* **123**, 161.

HARRIS, G. & LITTLETON, R.J. (1966) The effects of antigens and of PHA on rabbit spleen cell suspensions. *J. exp. Med.* **124**, 621.

HIRAMOTO, R., GOLDSTEIN, M.N. & PRESSMAN, D. (1960) Limited fixation of antibody by viable cells. *J. nat. Cancer Inst.* **24**, 255.

HIRSCHHORN, K., BACH, F., KOLODNY, R.L., FIRSCHEIN, I.L. & HASHEM, N. (1963) Immune response and mitosis of human peripheral blood lymphocytes *in vitro*. *Science (N.Y.)*, **142**, 1185.

HOCHWALD, G.M., THORBECKE, G.J. & ASOFSKY, R. (1961) A new technique for the demonstration of the synthesis of individual serum proteins by tissues *in vitro*. *J. exp. Med.* **114**, 459.

HOLT, L.J., LING, N.R. & STANWORTH, D.R. (1966) The effect of heterologous antisera and rheumatoid factor on the synthesis of DNA and protein by human peripheral blood lymphocytes. *Immunochemistry*, **3**, 359.

HUBER, H., WINKLER, H., REISER, G., HUBER, C., GABL, F. & BRAUNSTEINER, H. (1967) Eigenschaften und subcellulare lokalisation neugebildeter proteine menschlicher lymphocyten *in vitro* mit und ohne phytohamagglutininzusatz. *Klin. Wschr.* **45**, 204.

HULLINGER, L. & SORKIN, E. (1963) Synthesis of antibodies by blood leucocytes of the rabbit. *Nature (Lond.)*, **198**, 299.

LANDY, M., SANDERSON, R.P. & JACKSON, A.L. (1965) Humoral and cellular aspects of the immune response to the somatic antigen of Salmonella enteritidis. *J. exp. Med.* **122**, 483.

LING, N.R., SPICER, E., JAMES, K. & WILLIAMSON, N. (1965) The activation of human peripheral lymphocytes by products of staphylococci. *Brit. J. Haemat.* **11**, 421.

LISCHNER, H.W., PUNNETT, H.H. & DIGEORGE, A.M. (1967) Lymphocytes in congenital absence of the thymus. *Nature (Lond.)*, **214**, 580.

MARSHALL, J.D., EVELAND, W.C. & SMITH, C.W. (1958) Superiority of fluorescein isothiocyanate (Riggs) for fluorescent antibody techniques with a modification of its application. *Proc. Soc. exp. Biol. (N.Y.)*, **98**, 898.

MEUWISSEN, H., BACH, F., VANALTEN, P. & GOOD, R.A. (1967) Cellular responses in immunological deficiency states. *Fed. Proc.* **26**, 477.

MOLLER, G. (1961) Demonstration of mouse isoantigens at the cellular level by the fluorescent antibody technique. *J. exp. Med.* **114**, 415.

MILLS, J.A. (1966) The immunologic significance of antigen induced lymphocyte transformation *in vitro*. *J. Immunol.* **97**, 239.

NOWELL, P.C. (1960) Phytohaemagglutinin: an initiator of mitosis in cultures of normal human leukocytes. *Cancer Res.* **20**, 462.

OPPENHEIM, J.J., ROGENTINE, G.N. & TERRY, W.D. (1967) Transformation of human lymphocytes by monkey antisera to human immunoglobulins. *Fed. Proc.* **26**, 528.

PARENTI, F., FRANCHESCHINI, P., FORTI, G. & CEPELLINI, R. (1966) The effect of phytohaemagglutinin on metabolism and γ-globulin synthesis of human lymphocytes. *Biochim. biophys. Acta (Amst.)*, **123**, 181.

PULVERTAFT, R.J.V. (1964) Phytohaemagglutinin in relation to Burkitt's tumour (African lymphoma). *Lancet*, **ii**, 552.

RIPPS, C.S. & HIRSCHHORN, K. (1967) The production of immunoglobulin by human peripheral blood lymphocytes *in vitro*. *Clin. exp. Immunol.* **2**, 377.

ROBBINS, J.H. (1964) Tissue culture studies of the human lymphocyte. *Science (N.Y.)*, **146**, 1648.

ROBBINS, J.B., MOLES, E., RIMON, A. & SELA, M. (1967) Correlation between net charge of antigens and electrophoretic mobility of immunoglobulin M antibodies. *Nature (Lond.)*, **213**, 1013.

SCHEIDEGGER, J.J. (1955) Une micro-methode de l'immuno-electrophorese. *Int. Arch. Allergy*, **7**, 103.

SELL, S. (1967) Studies on rabbit lymphocyte *in vitro*. V. The induction of blast transformation with sheep antisera to rabbit IgG subunits. *J. exp. Med.* **125**, 289.

SELL, S. & GELL, P.G.H. (1965) Studies on rabbit lymphocytes *in vitro*. I. Stimulation of blast transformation with an antiallotype serum. *J. exp. Med.* **122**, 423.

SELL, S., ROWE, D.S. & GELL, P.G.H. (1965) Studies on rabbit lymphocytes *in vitro*. III. Protein, RNA and DNA synthesis by lymphocyte cultures after stimulation with phytohaemagglutinin, with staphylococcal filtrate, with antialloytpe serum, and with heterologous antiserum to rabbit whole serum. *J. exp. Med.* **122**, 823.

STAVITSKY, A.B. (1958) *In vitro* production of diphtheria antitoxin by tissues of immunised animals. *Brit. J. exp. Path.* **39**, 661.

STAVITSKY, A.B. (1961) *In vitro* studies of the secondary response. *Advanc. Immunol.* **1**, 211.

THORBECKE, G.J., HOCHWALD, G.M. & JACOBSON, E.B. (1962) Autoradiography of immunoelectrophoresis in the study of serum protein formation by mouse plasma tumours and a few other transplantable tumours. *Ann. N.Y. Acad. Sci.* **101**, 255.

TURNER, K.J. & FORBES, I.J. (1966) Synthesis of proteins by human leukocytes *in vitro*. II. Chemical characterization. *J. Immunol.* **96**, 926.

VAN FURTH, R., SCHUIT, H.R.E. & HIJMANS, W. (1966) The formation of immunoglobulins by human tissues *in vitro*. *Immunology*, **11**, 1.