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THE PRODUCTION OF IMMUNOGLOBULINS BY HUMAN PERIPHERAL BLOOD LYMPHOCYTES *IN VITRO*

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SUMMARY

We have demonstrated that the peripheral blood lymphocytes of normal humans show an increased production of immunoglobulins *in vitro* after non-specific stimulation by phytohaemagglutinin (PHA). The methods used include immunofluorescence and immune co-precipitation. Specific antigens to which the donor has been sensitized produce a similar effect in a smaller number of cells. The cells of persons with defects of immunoglobulin production can be stimulated by PHA to enlarge and divide, but their immunoglobulin content is abnormal and reflects the pattern of the circulating serum globulins. Preliminary results suggest that a portion of the immunoglobulin produced after either specific or non-specific stimulation of cells from sensitized donors behaves like specific antibody.

INTRODUCTION

Czitober, Gollerkeri & Dameshek (1960) demonstrated by the use of immunofluorescent techniques the presence of γ-globulin in peripheral blood lymphocytes of rabbits hyperimmunized to typhoid vaccine. Fluorescent staining of immunoglobulins within cultured human lymphocytes was subsequently demonstrated after stimulation of the cells by exposure to phytohaemagglutinin, antigens to which the cell donor was sensitized, foreign lymphocytes, or pokeweed extract (Bach & Hirschhorn, 1963; Elves *et al.*, 1963; The & Eijsvoogel, 1964; Douglas *et al.*, 1966). In a preliminary report, Bach & Hirschhorn (1963) showed by paper electrophoresis, immunoelectrophoresis and autoradiography that PHA-stimulated human lymphocytes could synthesize immunoglobulin. The synthesis of immunoglobulins was confirmed by precipitation of ^{14}C-labelled protein with specific antisera and sodium sulphate (Forbes & Turner, 1965; Parenti *et al.*, 1966). Tuberculin-stimulated cells from a sensitized donor also appeared to behave similarly (Cooperband, Nahmias & Kibrick,

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1964). Even in the absence of an added stimulant, normal human lymphocytes synthesized *in vitro* small amounts of IgG, IgA and IgM as demonstrated by fluorescence, immunoelectrophoresis and autoradiography (van Furth, 1964). Unstimulated chicken peripheral blood lymphocytes were also shown to produce IgM *in vitro* (Marinkovich & Baluda, 1966). Furthermore, co-precipitation experiments on cells from sensitized rabbits or from humans with an 'autoimmune' disease showed that a portion of the protein synthesized by the cells behaved as a specific antibody (Hulliger & Sorkin, 1963; Forbes, 1965). The production of specific antibody was also demonstrated by the Jerne plaque technique (Landy *et al.*, 1964; Kearney & Halliday, 1965; Möller, 1965).

Recently, however, Sell, Rowe & Gell (1965) reported that they were unable to demonstrate IgG synthesis in cultures of rabbit peripheral blood lymphocytes. We have, therefore, decided to re-examine *in vitro* protein production by human peripheral blood lymphocytes using fluorescent techniques and co-precipitation.

The results of this study confirm that there is an increase in immunoglobulin synthesis by peripheral lymphocytes following PHA stimulation and strongly suggest that a portion of this material behaves as a specific antibody.

MATERIALS AND METHODS

Subjects

Normal cells were obtained from healthy laboratory personnel and medical student volunteers of both sexes. Sensitized cells for special studies were obtained, preferentially, from individuals in the same groups, or if these were not available, from hospital patients having the requisite sensitivities. Cells were also obtained from agammaglobulinaemic subjects and their mothers.

Glass washing procedure

All glassware, caps and pipettes were cleaned by boiling in a 5% aqueous solution of 7X detergent (Linbro Chemicals, New Haven, Connecticut). This was followed by a series of rinses using tap water and then triple-distilled water. The pipettes and glassware were sterilized in a hot-air oven; the rubber-lined tube and bottle caps were autoclaved.

Culture procedures

Venous blood was drawn into heparinized syringes or plastic packs (Fenwal Transfer packs TA-2, 300 ml capacity). A total of 5000 units of phenol-free heparin was used for each 10 ml of whole blood collected. The blood was transferred to screw-capped 15-ml glass tubes and permitted to sediment for 1–2 hr at 37°C. The lymphocyte-rich upper portion of the plasma was carefully removed and mixed with an equal volume of Eagle's minimal essential medium modified for Spinner culture (MEM, Grand Island Biological Co., Grand Island, New York) which had been previously warmed to 37°C. The majority of the polymorphonuclear neutrophilic leucocytes and monocytes were removed by incubation of this mixture for 1 hr at 37°C in flat-sided glass prescription-bottles. Preparations containing up to 98% lymphocytes were thus obtained. The small numbers of polymorphs present did not adversely affect the results in any detectable way. The lymphocytes were washed three times with 10–20 ml of MEM at 150–200 *g* and resuspended in culture fluid

which consisted of MEM to which 20% calf serum, 1% 200 mM-L-glutamine solution, 100 units of penicillin G and 100 μg of streptomycin (Grand Island Biological Co.) were added. For all experiments in which protein production was being evaluated by fluorescence or immune co-precipitation, 'agamma' calf serum (Grand Island Biological Co.) was used. This serum is prepared by ammonium sulphate precipitation and contains little immunoglobulin. For the remainder of the antigen experiments, foetal calf serum (Grand Island Biological Co.) was used. There was no difference in the response of lymphocytes in replicate cultures using the two different sera as judged by mitotic rate or morphological alteration. All antibotics were omitted from the culture fluid when cells from a person with a history of allergy to penicillin were used. Each culture contained 750,000–1,000,000 lymphocytes/ml in a final volume of 4–6 ml. Each series of experiments was performed on aliquots of the same washed cell suspension.

Radioactive materials

Experiments utilizing radioactive labelling procedures were performed on cultures to which L-leucine-^{14}C (S.A. = 222 mCi/mM) or L-phenylalanine-^{14}C (S.A. = 333 mCi/mM) (New England Nuclear Corp., Boston, Massachusetts) were added in sufficient quantity to give a final activity of 1 μCi/ml in the culture fluid at the beginning of the culture period.

Stimulants

Phytohaemagglutinin M (PHA, Difco Laboratories, Detroit, Michigan) was added to cultures in the amount of 0·1 ml of freshly reconstituted solution per tube. Occasional lots of PHA were found to be heavily contaminated with *Bacillus subtilis* and tubes prepared from these lots were discarded even though stimulated cells and numerous mitotic figures were often present. Bacterial contamination was otherwise not a problem, even in cultures lacking antibiotics.

Antigens were added to the culture tubes in the following doses: penicillin G (PCN), 100 units; purified protein derivative of tuberculin (PPD), $2{\cdot}5 \times 10^{-3}$ mg; streptolysin O (SLO), 100 units; tetracycline base, 1×10^{-4} mg.

Harvesting for morphological studies

Mitoses were arrested in those cultures containing PHA by the addition of 0·1 ml of a 0·5 μg/ml solution of Velban (vinblastine sulphate, Eli Lilly and Co., Indianapolis) 2 hr before harvesting. All other tubes including the negative controls were similarly treated 4 hr before harvesting. At the time of harvesting, the cell suspensions were centrifuged at 150 *g* for 5 min, resuspended once in 1% sodium citrate and recentrifuged at 40 *g* for 5 min. The cells were then fixed in a 3:1 mixture of methanol and glacial acetic acid for 7 min, centrifuged for 5 min at 40 *g* and resuspended in fresh fixative.

Drops of the cell suspension were placed on glass cover slips, air-dried and stained with 0·5% orcein in 45% acetic acid. A total of 1000 cells from each tube was examined by phase contrast microscopy with a Zeiss photomicroscope and the percentage of stimulated cells was recorded. Morphological alterations interpreted as evidence of stimulation consisted of enlargement of the nucleus or a change in the quality of nuclear staining. Unstimulated cells ('small' lymphocytes) are characterized by a small, round, darkly-staining nucleus typical of heterochromatin, while stimulated cells show a light reticular nuclear

staining pattern typical of euchromatin and frequently have prominent nucleoli (see Fig. 1). Cells in mitosis are also considered as stimulated cells. The percentage of stimulated cells in the negative control culture was in each case subtracted from the percentage in the test cultures. It can be shown by χ^2 analysis that a difference between control and test cultures of fifty stimulated cells per 1000 sampled (5%) is highly significant. An increase of at least 5% compared to control values was therefore considered a positive response.

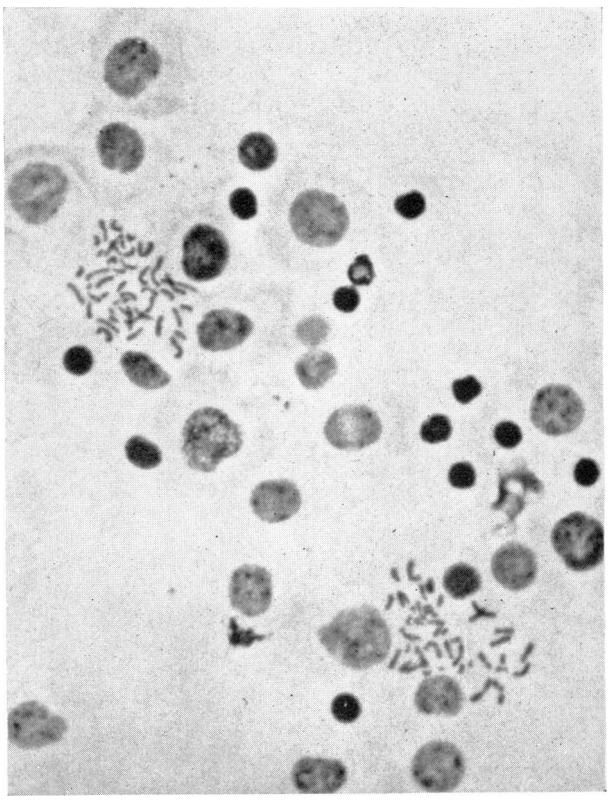


FIG. 1. Normal lymphocytes stimulated by phytohaemagglutinin after 3 days of culture. This field contains twenty-six stimulated cells, two of which are in mitosis. Nine unstimulated cells and several disintegrating cells are also present. Acetic-orcein stain, × 350.

Antigens and antisera

Antisera raised in goats and rabbits against IgG, IgM and IgA are commercially available both as the fluorescein-conjugated and the unconjugated forms (Beringwerke, Marburg am Lahn, Germany). These antisera have been absorbed with light chains and are, therefore, specific for the γ, μ and α H- (heavy) chains. Highly purified human IgG, and highly purified and absorbed antisera directed against IgG, IgM, H- (γ) chains of IgG, and κ type L- (light) chains, both fluorescein-conjugated and non-conjugated, which were prepared by Dr E. C. Franklin, were also used. Since no difference in the intensity, distribution, or

extent of fluorescent staining was observed when the highly specific antisera to IgG and IgM were compared to the commercial products, the latter were used for the majority of the experiments. The commercial antisera gave single precipitin arcs when diffused against whole human serum in micro-Ouchterlony plates or on agar gel electrophoresis. Controls for non-specific staining are discussed under 'Results'. Some other commercially available antisera to human immunoglobulins produced much more non-specific staining and were not employed. Goat anti-rabbit γ-globulin and rabbit anti-goat γ-globulin were purchased from Hyland Laboratories (Los Angeles, California).

Recrystallized bovine γ-globulin (Armour), and rabbit anti-bovine γ-globulin which had been thoroughly absorbed with human IgG, were used as controls for non-specific precipitation in one group of experiments. Ovalbumin and rabbit anti-ovalbumin, which did not cross-react with any other components of the culture system, were used for the same purpose in another group of experiments.

An antibody to the penicilloyl group prepared in rabbits by Dr Bernard Levine (Levine *et al.*, 1966a) was fluorescein-conjugated and rechecked for specificity against the penicilloyl group by Dr Michael Fellner.

Fluorescence studies

No Velban was added to cultures of cells intended for fluorescence studies. At the time of harvesting, the living unfixed cells were washed three times with 1–2 ml of MEM at 250 *g* and then incubated for 45 min at 37°C with the previously ascertained optimal volume of fluorescein-conjugated antiserum (usually 0·025 ml of the commercial product). The stained cells were again washed three times, air-dried on cover slips at room temperature and mounted in phosphate-buffered glycerol. The slides were examined on a Zeiss Ultraphot microscope equipped with an HBO 200 mercury lamp and a BG 12 excitation filter. A removable yellow barrier filter (Kodak K_2) was used for viewing the slides and for photography. All photographs were taken through a monocular tube with a Zeiss 35 mm camera attachment. Kodak High Speek Ektachrome film was used with exposure times of approximately 60 sec. A minimum of 500 lymphocytes was counted from each culture. In most cases, at least 1000 cells were scored for presence and intensity of fluorescent staining which was graded as 1+ to 4+.

Blocking experiments were performed similarly, but a two- to three-fold excess of the unconjugated antiserum was used to pretreat the cells for 45 min at 37°C before the fluorescein-conjugated antiserum was applied.

Attempts to stain smears of the cell suspensions after alcohol–acetic acid fixation resulted in uneven staining, often confined to areas of the cell membranes. In addition, quantitative studies on these fixed preparations were non-reproducible. This method of staining was therefore not employed.

Co-precipitation of newly-synthesized IgG

Samples consisted of: (1) extracts of lymphocytes which had been repeatedly frozen and thawed in medium containing no ^{14}C-labelled amino acids and then centrifuged at 600–1000 *g* for 10 min to remove insoluble particulate matter, (2) unconcentrated supernatants from the lymphocyte cultures, and (3) supernatants concentrated from ten to fifty times by dialysis against Carbowax (Union Carbide Co., New York).

Aliquots of the samples were mixed with carrier serum from the same individual who donated the lymphocytes. The IgG was precipitated by incubation of the mixture for 1 hr at 37°C with a slight excess of anti-IgG above that required for maximal precipitation of the carrier IgG. In most cases a ratio of 32 volumes of anti-IgG per volume of carrier serum was required. The supernatants from these precipitations did not contain significant amounts of IgG as judged by the failure to produce precipitin lines on micro-Ouchterlony plates when reacted with additional antiserum. The antigen–antibody precipitates were washed four times with ice-cold saline, dissolved in Hyamine (Packard Instrument Co., Downers Grove, Illinois) and transferred as completely as possible to 10 ml of a scintillation mixture (Bray, 1960).

Estimates of total cell proteins were obtained by overnight precipitation of other aliquots with 9 volumes of cold 10% trichloroacetic acid (TCA). These precipitates were washed four times with ice-cold 10% TCA before they were dissolved in Hyamine.

All samples were counted for 10 min in a Packard Tri-Carb Scintillation Counter having an 85% counting efficiency in the range of radioactivity employed. Background counts were insignificant compared to the sample counts in all cases except the zero time samples. Counts on zero time samples did not differ from background counts after corrections for non-specific precipitation had been applied.

Two types of controls were employed to correct for non-specific precipitation of radioactivity by antigen–antibody complexes. In the first series of experiments, identical aliquots of the sample–carrier serum mixture were treated with sufficient ovalbumin and anti-ovalbumin to yield a precipitate comparable in size to that of the IgG–anti-IgG precipitate. These precipitates were washed in saline, dissolved in Hyamine and counted. The counts so obtained were then subtracted from the counts recorded with the corresponding IgG–anti-IgG complex. In the second series of experiments, the sample-serum aliquots to be used for the immunoglobulin precipitations were first treated with bovine γ-globulin (BGG) and anti-bovine γ-globulin (anti-BGG) previously absorbed with human IgG. The supernatants were then removed and the human IgG was precipitated with the corresponding antiserum. No attempt was made to recover all the human IgG trapped in the BGG–anti-BGG precipitates although assays of a few washes from this precipitate showed that there could be a loss of up to one-third of the labelled IgG in this manner.

Controls for the TCA precipitation consisted of cultures which had been grown in medium to which leucine-^{14}C had been added only at the instant before harvesting. The counts obtained from these control precipitates were subtracted from the counts in the precipitates from cells which had the opportunity to incorporate the radioactive amino acid. All counts were corrected for background and machine efficiency and are expressed as counts/min/6×10^6 lymphocytes.

Allergy testing

Subjects were tested for sensitivity to PPD by the standard intradermal technique using first strength or intermediate strength material (Merck, Sharpe and Dohme, West Point, Pennsylvania). Penicillin allergy was ascertained by Dr Michael Fellner using skin tests with penicillin G and benzylpenicilloyl-polylysine (Sigma Chemical Co., St Louis) and by determination of haemagglutinating antibody titres (Levine, Fellner & Levytska, 1966). Subjects were considered sensitized to streptolysin O (SLO) if they had recently had a

documented streptococcal infection or had elevated ASLO titres. Tetracycline allergy was ascertained by intradermal injection of 5×10^{-3} mg of the antibiotic. Sensitivity was indicated by development of an immediate wheal-and-flare reaction which could be passively transferred to a normal subject by the patients' serum (Fellner & Baer, 1965).

RESULTS

Development of fluorescence with antisera to IgG, IgM and IgA

The fluorescence technique utilized, produced easily identifiable specific staining with negligible background fluorescence. Although some cellular debris, degenerated polymorphonuclear leucocytes, and occasional fibrin clumps were encountered, these could be easily distinguished from the well-preserved lymphocytes.

TABLE 1. Maximum percentage of normal lymphocytes stained by fluorescein-conjugated antisera to immunoglobulins

Stimulant added to culture	Incubation period (days)	Blocking Serum*	Specificity of antiserum			
			IgG (%)	IgM (%)	IgA (%)	Rabbit γ or goat γ (%)
None	0	—	0·3–0·5	0·3–0·5	0·1–0·5	0
None	3	—	≤5·0	≤5·0	≤5·0	0
PHA	2–3	—	75–95	75–95	75–95	0
PHA	2–3	Anti-IgG	0–5	75–90	75–90	—
PHA	2–3	Anti-IgM	75–95	0–5	75–90	—
PHA	2–3	Anti-IgA	75–95	75–90	0–5	—

* Added after harvesting.

With each of the antisera, diffuse cytoplasmic fluorescence was observed in large and small cells. Occasional cells had fluorescent spots in the otherwise unstained nuclear area. In the small, very intensely stained lymphocytes it was often difficult to define the nucleus (Fig. 2).

Those cells which were classified as non-fluorescent were almost invisible with the filter system employed and it was often necessary to remove the yellow barrier filter in order to ascertain by morphology and the presence of an intense blue autofluorescence that they were indeed lymphocytes (Figs. 7 and 8). The fluorescing cells examined in this manner showed a striking superimposition of green and blue (Fig. 4).

The number of lymphocytes staining with fluorescein-conjugated antisera to IgG, IgM and IgA showed a definite and reproducible variation with time. Cell suspensions examined shortly after collection showed only 0·3–0·5% of lymphocytes stained with anti-IgM and IgG and 0·1–0·5% stained with anti-IgA. Even after 3 days incubation in the absence of PHA, the cultures never contained more than about 5% of lymphocytes stainable with any of the three antisera.

Those cells cultured with PHA exhibited a markedly different pattern. There was little if any increase in fluorescence after 12 hr incubation, but by 36 hr the majority of cells

stained brightly with anti-IgM and moderately so with anti-A; the anti-IgG staining was weaker, but as diffuse. The anti-IgG staining steadily increased in intensity over the next 24 hr until by about 60 hr it had equalled the intensity of the IgM staining and was present in over 90% of the lymphocytes. The intensity and distribution of the IgA and IgM fluorescence was diminishing by 72 hr while that of the IgG was reaching its peak in up to 95% of the cells (Fig. 3). The maximal staining with anti-IgG occurred between 72 and 96 hr and then slowly faded.

The appearance of fluorescent cells in a series of PHA-stimulated cultures preceded the appearance of numerous large cells in the orcein-stained preparations (Fig. 6). In

TABLE 2. Percentage of stimulated lymphocytes on orcein-stained preparations

Stimulant added to culture	Incubation period (days)	Stimulated cells (%)
None	0	0·5–1
None	3	5·8
PHA	3	75–98

general, the fluorescent staining of the very large cells was less intense than that of the small and medium-sized lymphocytes, but this probably represents a dilutional effect due to the rapidly expanding cytoplasmic mass in the pre-mitotic phase.

FIGS. 2–8: excitation filter BG 12.

FIGS. 2, 3, 5 and 6: yellow barrier filter (K_2) in optical path.

FIG. 2. Phytohaemagglutinin-stimulated culture stained at 36 hr with anti-IgM. A small cell is intensely stained. × 720.

FIG. 3. Phytohaemagglutinin-stimulated culture stained at 72 hr with anti-IgG. A large lymphocyte is intensely stained. × 720.

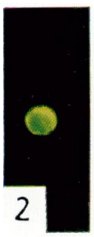
FIG. 4. Same preparation as in Fig. 3. A large, intensely stained cell is shown without the yellow filter. The blue autofluorescence and green specific fluorescence are superimposed. This cell has a somewhat eccentric nucleus which is occasionally observed in stimulated lymphocytes. × 1800.

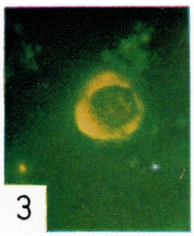
FIG. 5. Antigen-stimulated culture stained at 72 hr with anti-IgG. A stimulated cell shows cytoplasmic and 'nuclear' staining of moderate intensity. Five unstimulated cells at the left of the field are not fluorescent and barely visible. × 720.

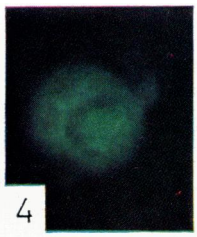
FIG. 6. Phytohaemagglutinin-stimulated culture stained at 36 hr with anti-IgM. The majority of lymphocytes are moderately fluorescent, although few have begun to enlarge. × 720.

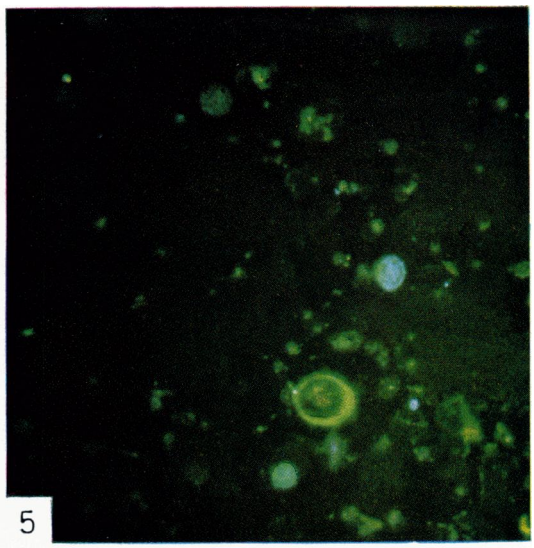
FIG. 7. Unstimulated culture stained at 72 hr with fluorescein-conjugated anti-IgG. No specific fluorescence is seen, although an occasional lymphocyte has enlarged. With the barrier filter in place, these cells were virtually invisible. This field also contains several autofluorescent granulocytes. × 720.

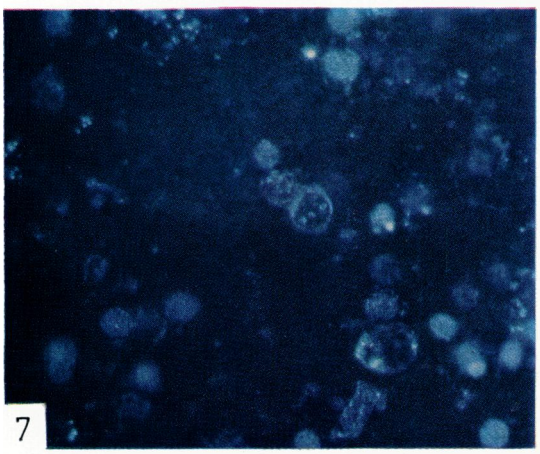
FIG. 8. Phytohaemagglutinin-stimulated culture incubated at 72 hr with unconjugated anti-IgG followed by fluorescein-conjugated anti-IgG. The specific fluorescent staining (seen in Fig. 4) has been completely abolished. Only blue autofluorescence is visible within stimulated lymphocytes. A fragment of granulocyte cytoplasm is adherent to one cell. × 1800.

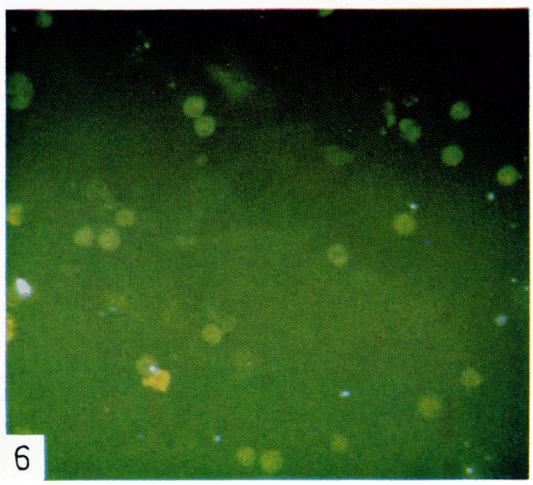


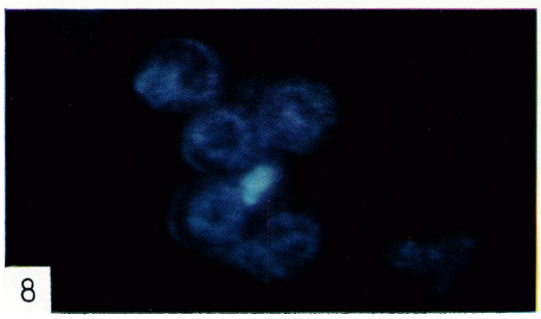












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The specific fluorescent staining could always be abolished by pretreatment of the cells with a sufficient excess of non-conjugated antiserum. Use of the smaller than optimal amounts in several early experiments resulted in the persistence of a few weakly-staining cells after fluorescent antiserum treatment. In no case did pre-incubation with an excess of antiserum directed against a different immunoglobulin fraction abolish or significantly decrease the subsequent fluorescent staining. There was, furthermore, absolutely no fluorescent staining of lymphocytes after 3 days of incubation with PHA when rabbit anti-goat or goat anti-rabbit γ-globulins were employed. The results are summarized in Table 1; values represent the extremes recorded at the peak of the response in multiple experiments on all individuals. The variation between replicate samples was less than 5% of the mean.

TABLE 3. Fluorescent staining after 3 days of culture with antigens

Stimulant added to culture	Specificity of antiserum		
	IgG (%)	IgM (%)	IgA (%)
Sensitized patients			
None	≤5	≤5	≤5
Antigen (PCN*, SLO, PPD, tetracycline)	5–35	5–35	5–35
Antigen (PCN†,)	25	55	35
Normal controls			
None	≤5	≤5	≤5
Antigen PCN, SLO, PPD, tetracycline)	≤5	≤5	≤5

* Two patients tested 1 and 7 years after the last clinical reaction.
† One patient tested 7 days after the last clinical reaction.

Phase contrast microscopy of the corresponding orcein-stained preparations revealed that comparable numbers of lymphocytes were undergoing enlargement or mitosis at these times (Table 2).

In two experiments with antiserum to κL-chains, however, only 35–40% of the PHA-stimulated lymphocytes present showed specific fluorescence, while more than 90% of these cells reacted with antisera directed against any of the three H-chains.

Fluorescence with antiserum to IgG, IgM and IgA in cells from sensitized individuals cultured with antigens (Table 3)

Cells from sensitized individuals showed qualitatively the same patterns of fluorescent staining as those of normal persons cultured with PHA, but to a lesser and more variable degree. There was also some dependence on the time elapsed since the last known exposure to the antigen. As with the PHA-stimulated cells, the fluorescence induced by exposure to the appropriate antigen could be specifically and completely blocked by pretreatment with the unconjugated antiserum. It was our impression that the time sequence of appearance

of fluorescent staining paralleled, but was somewhat more delayed than that observed with PHA (Fig. 5). We do not yet have sufficient data from a variety of antigen studies to determine if this is a general phenomenon. Comparable numbers of cells were observed to be responding in the corresponding cultures examined by phase contrast microscopy after 5 days of culture with these antigens. No more than half the lymphocytes present in any culture responded to antigenic stimulation as judged by fluorescent staining or morphological alteration.

Fluorescence in cells from agammaglobulinaemic patients after PHA stimulation (Table 4)

Although a normal percentage of the peripheral blood lymphocytes of eight patients with congenital agammaglobulinaemia showed enlargement and mitotic activity after 3 days of incubation with PHA, the cells were virtually devoid of immunoglobulin of any type.

TABLE 4. Percentage of fluorescing cells after 2–3 days of culture with phytohaemagglutinin

Subjects	No.	Specificity of antiserum		
		IgG (%)	IgA (%)	IgM (%)
Congenital agammaglobulinaemics (sex-linked and autosomal)	8	<1	<1	<1
Mothers of sex-linked agammaglobulinaemics	5	50–60	50–60	50–65
Mothers of autosomal agammaglobulinaemics	2	80–95	80–95	80–95
Dysglobulinaemics (normal IgG and IgM, absent IgA)	2	85–95	<1	85–95

Mothers of males with sex-linked agammaglobulinaemia are heterozygous for the defective gene, and might be expected to have two cell populations of approximately equal size, one of which is normal and one of which is agammaglobulinaemic. In fact, only 50–65% of the lymphocytes from such women contained immunoglobulin after stimulation with PHA (Table 4), a finding consistent with the prediction of the Lyon hypothesis (Lyon, 1962). Cells from mothers of patients with familial autosomally inherited agammaglobulinaemia behaved in culture like cells from normal individuals and did not show two cell populations.

The cultured cells of two patients who had normal or elevated serum IgG and IgM levels, but lacked IgA, fluoresced normally with antiserum to IgG and IgM, but there was no fluorescence with antiserum to IgA. This suggested that a quantitative comparison of serum levels of the γ-globulin fractions and the percentage of lymphocytes which could be shown to contain these fractions would be of interest. Accordingly, cells from an atypical agammaglobulinaemic patient, who was known to be producing decreased levels of all three major immunoglobulins, were cultured in the presence of PHA. The cells were treated as previously described except that the fluorescence slides were coded and counted by one of us (C.S.R.) without prior knowledge of which fluorescein-conjugated antiserum had been

applied. Dr H. Fudenberg then provided us with the levels of circulating immunoglobulins expressed as percentages of the normal serum levels. Comparison of these numbers shows an excellent correlation between the two sets of measurements (Table 5).

TABLE 5. Comparison of the percentage of lymphocytes containing immunoglobulins with serum levels in a dysgammaglobulinaemic subject

	Specificity of antiserum		
	IgG	IgM	IgA
Percentage of cells stained	31	<1	40
Percentage of normal level present in serum	20–30	<3	40–50

Incorporation of L-*leucine-*^{14}C *into proteins precipitable by anti-IgG and TCA*

In order to demonstrate that the immunoglobulin which appears within PHA-stimulated cells is actually synthesized during the culture period, we examined groups of cultures with and without PHA for IgG synthesis and protein production. The results of three experiments utilizing specific anti-IgG precipitation of cell extracts are shown in Fig. 9.

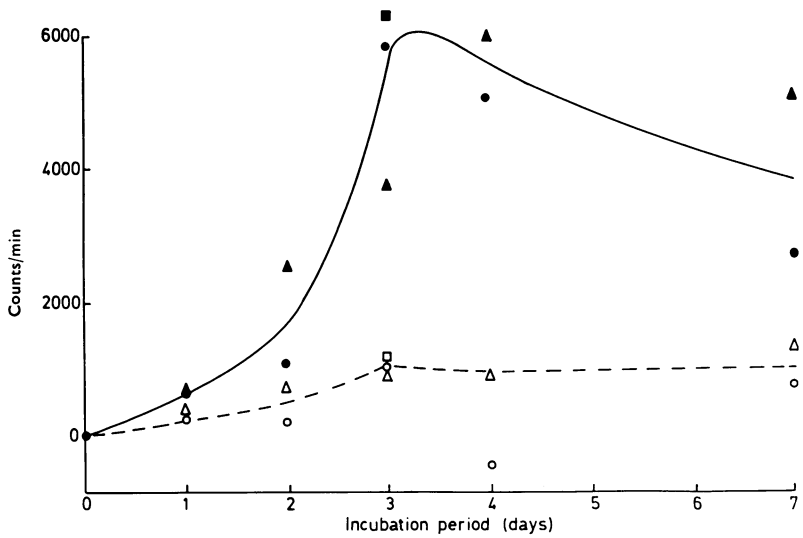

FIG. 9. Incorporation of leucine-^{14}C into IgG by normal lymphocytes. Results are expressed as counts/min/6×10^6 lymphocytes. ●, ▲ and ■, with PHA; ○, △ and □, without PHA. The same symbol has been used for each group of experiments on each subject.

In all experiments the peak intracellular content of labelled IgG was found between days 3 and 4 in agreement with fluorescence findings. The TCA-precipitable, intracellular labelled protein was also maximal at days 3 to 4.

In all series, there was a marked difference between the PHA-stimulated cells and the negative controls with respect to IgG and protein content. Table 6 compares the incorporation of L-leucine-^{14}C into IgG and total TCA-precipitable proteins in the presence and absence of PHA for two groups of cultures. The addition of PHA to these two groups produced more than a five-fold increase in the content of newly synthesized protein and a five- to seven-fold increase in the content of newly synthesized IgG at the peak of the response.

TABLE 6. Incorporation of L-leucine-^{14}C into IgG and other lymphocyte proteins

Experiment	Counts/min/6 × 10^6 cells		Percentage of protein counts attributed to IgG
	IgG*	Protein†	
Without PHA			
1	765	25,424	3·0
2	1082	29,976	3·6
With PHA			
1	5859	109,688	5·3
2	6388	142,224	4·5

* Precipitated by anti-IgG.
† Precipitated by TCA.

The relative amounts of IgG and protein synthesized, however, were not markedly affected by the addition of PHA, the percentage of counts in the TCA-precipitable material which could be ascribed to IgG varying between 2·2 and 5·3% for all cultures. No attempt was made to isolate or identify the non-immunoglobulin proteins synthesized or to measure the labelled protein in the cellular debris remaining after freeze–thaw extraction.

TABLE 7. Effect of controls on calculated IgG content

Experiment	Control		Counts/min in IgG
1	Ovalbumin–anti-ovalbumin	Separate tubes	5659
	BGG–anti-BGG	Separate tubes	5380
2	BGG–anti-BGG	Same tube	5859
3	BGG–anti-BGG	Same tube	6388

The unconcentrated culture fluids did not consistently contain a significantly increased amount of IgG compared to the controls. Only when the supernatants were concentrated fifty times could we demonstrate an increase in newly-synthesized IgG comparable to that in the cell extracts. We, therefore, elected to abandon supernatant analysis and utilize cell extracts exclusively, even though there was a considerable loss of labelled IgG into the supernatant fluid.

There was no great difference in the number of counts attributed to IgG with the two types of controls used to correct for non-specific precipitation of labelled material. By either method and with either antigen–antibody complex the cell extracts contained approximately 6000 counts/min/6×10^6 lymphocytes above control values at the peak of IgG synthesis (Table 7).

Quantitative estimation of IgG synthesis

One may calculate the approximate quantity of new IgG which the human lymphocyte contains, assuming that human IgG has the same amino acid composition (7·4% leucine) as rabbit γ-globulin (Crumpton & Wilkinson, 1963), and ignoring the increment in the size of the pool of unlabelled leucine due to the calf serum added.

Calculation:

μg of newly synthesized IgG per 10^6 lymphocytes

$$= \frac{a}{b} \times \frac{1}{x} \times \frac{1}{y}$$

where

a = average counts per minute corrected for background, machine efficiency and dilution factors

b = counts/min/μg of leucine-^{14}C

$$= \frac{\text{specific activity of leucine-}^{14}\text{C (mCi/mM)} \times \text{counts/min/mCi}}{\text{mg leucine/mM} \times 10^3}$$

$$= \frac{222 \times 2{\cdot}22 \times 10^9}{131{\cdot}7 \times 10^3}$$

$$= 3{\cdot}74 \times 10^6$$

x = fraction of the leucine pool which is ^{14}C-labelled

$$= \frac{\text{mg leucine-}^{14}\text{C added}}{\text{mg leucine present initially} + \text{mg leucine-}^{14}\text{C added}}$$

$$= \frac{5{\cdot}9 \times 10^{-4}}{(5{\cdot}9 \times 10^{-4}) + (4{\cdot}19 \times 10^{-2})}$$

$$= 1{\cdot}3 \times 10^{-2}$$

y = fraction of IgG which is leucine

For the PHA-stimulated cells at 3–4 days, the average IgG content is, therefore:

$$= \frac{5970}{3{\cdot}74 \times 10^6} \times \frac{1}{1{\cdot}3 \times 10^{-2}} \times \frac{100}{7{\cdot}4}$$

$$= 2{\cdot}8\ \mu\text{g}$$

By a similar calculation, the intracellular IgG produced in the first 24 hr by 6×10^6 unstimulated cells is found to be 0·15 μg. Using Nossal's estimate that 5×10^8 lymphocytes weigh 1 g (Nossal & Mäkelä, 1962) and assuming a uniform rate of production during the first 24 hr, each gram of unstimulated cells synthesizes

$$\frac{0{\cdot}15}{24} \times \frac{5 \times 10^8}{6 \times 10^6} = 0{\cdot}5\ \mu\text{g of IgG/hr}$$

Demonstration of antigen binding by fluorescence techniques

Evidence for the production of complete γ-globulin molecules was provided by the suggestion of specific antibody activity in the newly-synthesized material. Lymphocytes from four patients who had serum sickness reactions to penicillin were studied by immunofluorescent techniques. The cells were cultured with PHA, with 100 units of penicillin, or with no additives in penicillin-free medium. All of these subjects had positive immediate wheal-and-flare skin reactions and elevated levels of circulating haemagglutinating antibodies to penicillin or its breakdown products. At the end of the culture period all cells were washed thoroughly. Those which had been grown in the presence of penicillin were

TABLE 8. Comparison of percentage of cells binding penicillin with percentage of cells containing immunoglobulins after 3 days of culture

Stimulant added to culture initially	Added at 72 hr	Specificity of fluorescein-conjugated antiserum			
		BPO (%)	IgG (%)	IgA (%)	IgM (%)
Non-allergic controls (4)*					
Nothing	Nothing	0	≤5	≤5	≤5
PCN	Nothing	0	≤5	≤5	≤5
PHA	PCN	0	—	—	—
PHA	Nothing	0	80–90	75–80	80–85
Allergic patients, 1 and 7 years after serum sickness (2)*					
Nothing	Nothing	0	<5	<5	<5
PCN	Nothing	3–7	15–20	15–20	15–20
PHA	PCN	11–20	—	—	—
PCN	Anti-BPO	0	—	—	—
PHA	PCN+anti-BPO	0	—	—	—
PHA	Nothing	0	85–90	85–90	80–85
Allergic patients, 7 and 10 days after onset of serum sickness (2)†					
Nothing	Nothing	0	≤5	≤5	≤5
PCN	Nothing	18–20	20–25	30–35	50–55
PHA	PCN	54–70	—	—	—
PCN	Anti-BPO	0	—	—	—
PHA	PCN+anti-BPO	0	—	—	—
PHA	Nothing	0	85–90	80–85	85–90
Nothing	PCN	<5	—	—	—

* No fluorescence at zero time with fluorescein-conjugated anti-BPO. Antisera to IgG, IgM and IgA stained 0·1–0·3% of the cells in the initial suspension.

† No fluorescence at zero time with fluorescein-conjugated anti-BPO. Maximal staining of cells in the initial suspension was as follows: anti-IgG 0·3%, anti-IgA 3·9%, anti-IgM 5·0%.

incubated with fluorescein-conjugated antiserum to benzylpenicilloyl-BGG (BPO) then washed and dried in the same manner as the other slides prepared for fluorescence microscopy. Those which had not been exposed to penicillin during the culture period were preincubated with 100 units of penicillin for 45 min at 37°C after harvesting and then washed

prior to fluorescent staining. A number of controls were also used. The results are summarized in Table 8.

There was binding of penicillin to the lymphocytes of the penicillin allergic patients whether the cells were stimulated by penicillin or by PHA. In fact, the PHA appeared to be a better stimulus to production of the specific penicillin-binding material than the antigen itself. There was no non-specific staining with the conjugate and the specific staining could be completely blocked by pretreatment with non-conjugated antiserum. The cells from non-allergic control patients did not bind penicillin. PHA-stimulated cells from sensitized subjects never exhibited fluorescence with antiserum to the BPO group unless they had been pre-incubated with penicillin. The two patients who recently had a severe allergic reaction showed a higher percentage of circulating cells containing immunoglobulins at zero time than did normal subjects or allergic subjects without recent exposure to the antigen; they also showed a slightly greater percentage of cells containing immunoglobulin after 3 days exposure to penicillin and a higher percentage of cells containing penicillin-binding material after stimulation with either penicillin or PHA than did allergic subjects not recently exposed to penicillin.

DISCUSSION

Although previous studies have provided evidence suggestive of immunoglobulin synthesis by peripheral blood lymphocytes, this suggestion has not been uniformly accepted. This role in the immune response has traditionally been ascribed to the plasma cell, largely on the basis of studies in solid lymphoid tissues where it undoubtedly is associated with antibody production. The origin and ancestry of the plasma cell are still in doubt, however, and numerous studies have suggested that interconversions may occur between cells classified by morphological criteria as lymphocytes, reticulum cells and plasma cells. In diseases characterized by excessive production of protein, such as Waldenstrom's macroglobulinaemia or certain lymphatic leukaemias, abnormal proteins have been demonstrated within 'lymphoplasmacytoid' cells (Zucker-Franklin, Franklin & Cooper, 1962) or abnormal lymphocytes (Gamble & Cutting, 1966) which are present in the circulation. Gowans has shown that small lymphocytes appear to be responsible for graft-versus-host reactions in the course of which the donor cells are transformed into large pyroninophilic basophilic cells having little endoplasmic reticulum (Gowans & McGregor, 1965). Stimulation of peripheral blood lymphocytes by either specific antigens or non-specific stimulants such as PHA lead to conversion of many of the cells to morphologically similar pyroninophilic forms which resemble, but are not identical to, plasma cells. Recent studies with an extract from the pokeweed *Phytolacca americana*, another potent non-specific mitogen, have shown conversion of cultured lymphocytes to cells having even more rough endoplasmic reticulum than the cells stimulated by PHA. These pokeweed-stimulated cells are also stainable by fluorescein-conjugated anti-human globulin (Douglas *et al.*, 1966). Whether or not the peripheral lymphocyte stimulated *in vitro* to assume this morphology is truly analogous to Gowan's pyroninophilic cell and what its ultimate fate would be if retained within its host is not clear at this time.

The small lymphocyte of the peripheral blood is a long-lived cell possibly surviving *in vivo* from months to years before undergoing mitosis (Buckton *et al.*, 1962; Norman *et al.*,

1965; Nowell, 1965). This long life span, in combination with its freedom to migrate through or near all the possible areas of antigen recognition and antibody production, make the small lymphocyte a good candidate for the immunological memory cell in the intact animal. If this is true, it would not be surprising that the majority of these cells normally circulate in an immunologically competent, although repressed state. If these cells are responsible for immunological memory, the best candidate for a 'memory molecule' would be the combining site of an antibody immunoglobulin. Gell and Sell have, in fact, reported evidence for the presence of γ-globulin within rabbit peripheral blood lymphocytes by showing an additive stimulation by monospecific anti-allotype sera of sub-populations of cultured lymphocytes from rabbits heterozygous for γ-globulin allotypes (Gell & Sell, 1965).

Sell *et al.* (1965), however, were unable to demonstrate IgG synthesis by rabbit peripheral blood lymphocytes *in vitro* with or without stimulation. On the other hand, they presented strong evidence for the ability of these cells to synthesize IgG *in vivo* (Gell & Sell, 1965). The apparent presence of only one or the other IgG allotype in sub-populations of cells despite the exposure of all the lymphocytes from heterozygous animals to both allotypes *in vivo*, strongly implies *in vivo* synthesis of IgG by these cells.

Sell *et al.* (1965) have discounted previous work consistent with *in vitro* IgG synthesis by peripheral blood lymphocytes because the donor animals or humans were hyperimmunized, infected, or stimulated by specific antigens. These objections do not apply to our results with PHA. They have discounted other work because the preparations contained an occasional plasma cell or produced only small amounts of IgG. Although we never found true plasma cells in our initial suspensions, it is conceivable that an occasional cell was missed. However, these would have had to perform Herculean feats of replication and synthesis if they were the only sources of immunoglobulin in our cultures. There is no reason to doubt van Furth's conclusions merely on the basis of the small quantity of immunoglobulin synthesized by his unstimulated cell cultures (van Furth, 1964). We have demonstrated a marked difference in the IgG production between the PHA-stimulated and non-stimulated cells.

While the difficulties of Sell *et al.* (1965) in demonstrating immunoglobulin production by rabbit peripheral blood lymphocytes *in vitro* may reflect basic differences in species, it is far more likely that immunoglobulin production is a relatively minor synthetic activity of rabbit cells as well as human ones. These authors added a much smaller quantity of [^{14}C]-amino acid to their cultures than we did and permitted it to remain in contact with the cells for only 24 hr before harvesting. As they did not state the specific activity of the lysine-^{14}C employed, we cannot compare the sizes of the respective amino acid pools available to the lymphocytes. The presence of many other labelled materials of varying sizes in freeze–thaw extracts of cells tends to obscure the presence of small amounts of immunoglobulin in the eluates from Sephadex columns. Finally, the lack of specificity in their immunofluorescence reagents prevented these authors from drawing any conclusions from their fluorescence studies.

Our fluorescence experiments clearly indicate that the human peripheral blood lymphocyte stimulated *in vitro* accumulates significant amounts of IgG, IgM and IgA in its cytoplasm. The controls for fluorescence are summarized in Table 9. The specificity of the staining has been demonstrated by blocking and cross-blocking experiments and by the fact that the cells fail to stain with absorbed antisera directed against the serum globulins

of other species. Since these antisera are produced in goats and rabbits, as are the antisera to human immunoglobulins, the lack of staining also shows that rabbit and goat serum proteins do not adhere non-specifically to human lymphocytes. The possibility that immunoglobulins are merely stored in the lymphocytes is unlikely because of the virtual absence of fluorescent staining initially and the sequential appearance of staining in the stimulated cultures. Non-specific adherence of circulating γ-globulins to the cells is ruled out by the lack of staining observed in a significant portion of the cells of mothers of sex-linked agammaglobulinaemic subjects. Another convincing control is the fact that less than half of normal cells stain with antiserum to κ chains (despite the exposure of all the cells to circulating γ-globulin with κ-specificity *in vivo*). It is equally improbable that this represents material ingested from the culture medium by the transformed cells since very little immunoglobulin, especially IgG, is present in the culture fluid. Moreover, it is impossible to explain the dissociation in large cell formation and immunoglobulin content in the cells of the atypical agammaglobulinaemic patients' cells by this means.

TABLE 9. Summary of controls for fluorescent staining

Observation	Antibody specificity of conjugated antisera	Lack of non-specific adherence of conjugated antisera	Lack of non-specific adsorption or storage of circulating immunoglobulins
Blocking and cross-blocking experiments	×		
No staining with antiserum to rabbit or goat globulins	×	×	
No IgA staining in patients without IgA	×	×	
Lack of staining of normal cells by antiserum to BPO	×	×	
No staining in agammaglobulinaemic cells		×	
Staining of sub-populations of cells in mothers of agammaglobulinaemic patients		×	×
Staining of only a portion of normal cells by antiserum to κ chains		×	×
Absence of staining initially with sequential development of fluorescence			×

The presence of fluorescent spots associated with the nucleus of some of the larger stimulated cells is an interesting incidental finding. Intranuclear inclusions of proteinaceous material have been reported in cells from individuals with disorders characterized by excessive production of γ-globulin such as multiple myeloma, macroglobulinaemia (Waldenstrom's) and reactive plasmacytosis. Brittin, Tanaka & Brecher, (1963) have postulated that this represents intranuclear protein synthesis. Zucker-Franklin, however, provides an alternate explanation with electron-micrographs which show that such cells may contain secretory

products within the cisternae of the endoplasmic reticulum which extend into the perinuclear space. This may become so distended that it bulges into the nucleoplasm invaginating the inner nuclear membrane before it and producing a picture of an intranuclear inclusion bounded by a single membrane (Zucker-Franklin, 1965). We may be observing a similar phenomenon in our stimulated cells.

The co-precipitation data confirm that synthesis of material having the antigenic determinants of IgG is occurring in the cultured normal lymphocytes. Using either replicate controls or pretreatment of the identical samples to correct for non-specific precipitation, all samples show a comparable and significant increase in IgG synthesis following phytohaemagglutinin stimulation.

The amount of IgG synthesized in our cultures is in reasonable agreement with other published data. Van Furth states that production of IgG by his cultures of unstimulated monkey spleen lymphocytes approximated 1 μg of IgG/hr/g of wet tissue during the first 6 hr and then declined. Allowing for differences in species, site of origin of cells and differences in the culture procedures, our average IgG production of approximately 0·5 μg/hr/g of unstimulated cells for the first 24 hr is in good agreement. The maximal IgG production by the stimulated cells is in line with data from other investigators which have been reviewed by Van Furth (1964).

Our quantitative estimates of total protein production by PHA-stimulated cells at 24 hr agree well with those of Bach & Hirschhorn (1963) who found that the cells were capable of synthesizing approximately 1% of their own weight in TCA-precipitable material by that time. No attempts were made in the present study to follow the initial phases of protein production when it became obvious that the principal period of immunoglobulin synthesis occurred at about the 3rd day of culture.

Our calculated estimates of IgG and protein production are by no means maximum values. Both values are diminished by the amount of newly-synthesized material released into the supernatant fluid during the culture period and the amount of material which was broken down. Cooperband and co-workers have found that there is a considerable quantity of IgG released into the supernatant culture fluid (Cooperband *et al.*, 1966). There was also loss of IgG in the samples from which the BGG–anti-BGG complexes had been precipitated due to trapping within the control precipitate. In addition, there was no attempt to evaluate the amount of new protein incorporated into the particulate cellular debris remaining after the extraction procedure. On the other hand, our values for IgG synthesis may have been elevated slightly if the use of a single BGG–anti-BGG precipitation was inadequate in removing labelled non-IgG material before precipitation of IgG. Since the calculated maximum content of newly-synthesized IgG was the same whether we performed the control non-specific precipitations in the same tubes used for IgG or in separate tubes, we feel strongly that these control values are correct.

Additional evidence for the synthesis of immunoglobulin was provided by preliminary immunoelectrophoretic experiments showing the presence of labelling over the IgG arcs, both with anti-human serum and antiserum to the specific immunoglobulin fraction. Several other lines, including α_2 macroglobulins were also labelled. Experiments are now in progress to determine which other proteins are being synthesized.

The objection could still be raised that the synthesized material may represent only a portion of the IgG molecule bearing the antigenic determinants and that this material

happens to migrate with the immunoglobulins on immunoelectrophoresis. Evidence for the production of intact immunoglobulin molecules is provided, however, by the fluorescence studies on penicillin-allergic patients. An increase in material behaving like specific anti-penicillin antibody could be demonstrated within the cells after stimulation with either penicillin or PHA. Since this material was not demonstrable at the beginning of the culture period and the culture medium contained none of the patient's serum, PCN binding could be due neither to adherence of circulating antibody nor absorption from the culture fluid. By analogy with the IgG results, this penicillin-binding material appearing at the peak of immunoglobulin production may be presumed to have been newly synthesized. While these are admittedly only preliminary observations, they are quite suggestive of the presence of antibody specificity in the immunoglobulin produced by the peripheral blood lymphocytes. The surprisingly high percentage of circulating lymphocytes capable of producing this antibody-like material after PHA stimulation apparently reflects the 'hyper-immunized' state of individuals following a serum sickness reaction.

If 60–70% of an individual's circulating lymphocytes may be capable of producing specific antibody to a single antigen at the height of a secondary response, as this data suggests, Burnet's clonal selection hypothesis appears to be mathematically improbable for the peripheral blood lymphocyte after PHA stimulation as far as production of a single type of specific antibody is concerned. The likelihood, however, that one peripheral blood lymphocyte can respond to more than one antigen is supported by a series of experiments utilizing mixtures of antigens to which the donor was sensitized. We have found (Hirschhorn & Ripps, 1965) that mixtures of antigens stimulated greater numbers of lymphocytes than any one antigen, but the effect was considerably less than additive. This would suggest that each responding lymphocyte is capable of recognizing more than one antigen, but not all antigens.

Whether or not stimulation of peripheral lymphocytes by specific antigens leads to production of immunoglobulins with only one antibody specificity is not yet clear. This was suggested by an early report of Elves *et al.* (1963), but more recently Hiramoto & Hamlin (1965) have reported that a large proportion of the antibody-containing spleen cells from immunized guinea-pigs contained immunoglobulins directed against two determinants on the immunizing antigen.

In the present study, we have shown by specific immunofluorescent staining that the majority of normal peripheral lymphocytes contain all three major classes of immunoglobulins after PHA stimulation. Other workers have recorded production of more than one class of immunoglobulin by a minority of cells from the lymphoid tissues of hyper-immunized animals using serological and paired immunofluorescent techniques (Attardi *et al.*, 1959; Nossal, 1962; Pernis *et al.*, 1964; Pernis *et al.*, 1965; Colberg & Dray, 1963). Small numbers of blastlike cells in the germinal centres of unstimulated human lymphoid tissues have also appeared to contain more than one kind of immunoglobulin, H-chain, or L-chain (Mellors & Korngold, 1963; Pernis & Chiappino, 1964; Chiappino & Pernis, 1964; Bernier & Cebra, 1965). Comparison of the results of PHA stimulation and antigen stimulation in our cultures shows that PHA is a far more potent stimulant to immunoglobulin production than any specific antigen in both normal and sensitized humans. This was also the conclusion of Forbes (1965).

The circulating peripheral blood lymphocyte apparently has two levels of differentiation

with respect to its ability to synthesize immunoglobulins. On the one hand, most cells retain their ability to produce H-chains of all three major classes (α, γ and μ) which is activated when a potent non-specific stimulus to protein production is applied. On the other hand, the differentiation for production of one or the other type of L-chain (κ and λ) seems to be fixed and limited to sub-populations of the circulating lymphocytes. It is possible that the same is true for the subtypes of IgG heavy chains (e.g. We, Vi, etc.)

The ability of PHA to stimulate production of several types of immunoglobulin within a single cell may be related to its apparent ability to derepress a great number of inactive genes with a subsequent synthesis of many of the proteins which the cell is capable of producing, among them the immunoglobulins. This would explain why IgG constitutes only a minor percentage of the newly-synthesized intracellular protein both in the absence and presence of PHA. Pogo, Allfrey & Mirsky (1966) recently demonstrated a rapid increase in the rate of acetylation of histones when PHA was added to cultures of peripheral lymphocytes. This may be an early indication of gene activation in the stimulated cells.

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ADDENDUM

After this manuscript was completed, a report by Turner & Forbes (1966) appeared which also confirms that the human peripheral blood lymphocyte is capable of synthesizing IgG and IgM after stimulation with phytohaemagglutinin. These investigators used specific co-precipitation, immunoelectrophoresis, autoradiography and DEAE-cellulose chromatography. Their autoradiographic patterns are essentially identical with the ones produced in our preliminary experiments.

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