THE EFFECTS OF ANTIGENS AND OF PHYTOHEMAGGLUTININ ON RABBIT SPLEEN CELL SUSPENSIONS

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Phytohemagglutinin (PHA) was first shown to be an initiator of mitosis in cultures of normal human leukocytes from peripheral blood (1). Evidence has been presented (2) that the responding cells from human blood had the characteristics of lymphocytes and were derived from a nondividing population. This has been confirmed by other studies (3, 4) which showed that small lymphocytes transformed into a more primitive cell capable of division, and similar results have been described with rabbit peripheral blood cells (5).

The present studies were concerned with the effects of PHA on the stimulation of cell proliferation and antibody production by rabbit spleen cells and comparison with the effects of antigens which have been shown to stimulate DNA synthesis in cell suspensions from the spleens of preimmunized rabbits (6).

The mechanism of action of PHA is not clear although it has been suggested that it works like an antigen (7) but there is no direct evidence for this except for the fact that the responding cells have the morphological characteristics of cells potentially capable of antibody production. While it has been claimed that human cells, stimulated by PHA, can produce antibodies (8, 9) and that rabbit lymph node explants can produce a specific anamnestic response to antigens in response to PHA (10) no evidence of immunoglobulin synthesis was found in suspensions of rabbit peripheral blood cells responding to this material (11).

Materials and Methods

Animals.—New Zealand white rabbits weighing at least 2 kg were obtained from Purdey's Farm, Rochford, Essex, England.

Antigens.—Human serum albumin (HSA) and human γ-globulin (fraction G4) (HGG) were obtained from the Lister Institute of Preventive Medicine, London, and sheep red cells (SRC's) were supplied in Alsever solution by Burroughs Wellcome and Co., London.

Phytohemagglutinin (PHA).—Bacto-phytohemagglutinin P was obtained from Difco Laboratories, Inc., Detroit. It was stored dry at 4°C and solutions were prepared immediately before use. Material of the same batch number was used for all experiments reported here.

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Isotopes.—Thymidine-C^{14} at 30.4 mc/mM and thymidine-H^3 at 2 to 4 c/mM were obtained from the Radiochemical Centre, Amersham, England.

Medium.—Eagle's minimum essential medium, concentrated tenfold was obtained from Burroughs Wellcome and Co. Bicarbonate was added to adjust the pH to 7.2, and 10 mg streptomycin and 10,000 units of penicillin were added to 100 ml of medium, which was fortified with 15% pooled, normal rabbit serum, obtained by cardiac puncture.

Immunization.—The rabbits were immunized by a 4 wk course of 16 intravenous injections of alum-precipitated proteins, to a total dose of 20 to 40 mg, or a 2 wk course of 6 intravenous injections of SRC's to a total dose of 0.6 ml packed cells. Booster injections of 2 mg protein or 0.1 ml packed SRC's were given 2 to 3 days before killing.

Diffusion Chambers.—Millipore membranes of rated pore size 0.3 to 1 μ were obtained from Millipore Filter Corporation, Bedford, Massachusetts, through Thermal Control Ltd., Hove, Sussex, England. Membranes were cut in rectangles approximately 1 $\times$ 1.4 cm, and two rectangles were approximated and three sides sealed by dipping into a thin film of acetone. The chambers did not affect the condition of culture and their presence did not affect DNA synthesis of cells cultured outside them. In histological sections of chambers of varying pore size, no evidence of cell penetration, from within or without, was demonstrated.

Preparation of Spleen Cell Suspensions.—The suspensions were prepared by methods described by Dutton and Eady (6). Triplicate suspensions of 10^7 cells in 1.5-ml medium were incubated in 10-ml beakers at 37°C in an atmosphere of 5% carbon dioxide in oxygen. Antigens or PHA were added initially and the rate of DNA synthesis was measured by the incorporation of thymidine-C^{14} at 30.4 mc/mM into the cells, as counts per minute (CPM) per culture. For C^{14} counting, cells were washed in ice cold saline 3 times, and then dissolved in 6 N HCl and made up to 1.0 ml. 0.1 ml was plated on cover slips for gas-flow counting. In all experiments, the values reported represent the means of triplicate cultures which did not vary by more than 15%.

Preparation of Peritoneal Exudates.—The method of obtaining these cells from the peritoneal cavity of rabbits has been described in detail (12). The majority of cells which adhered strongly to glass after incubation for several hours had a morphology characteristic of macrophages and were not synthesizing DNA under the experimental conditions used. Therefore, in this report, these preparations of peritoneal exudate cells will be called macrophages.

Radioautography.—After incubation in 5 μc/ml of thymidine-H^3, radioautographs were prepared after washing cells once in ice cold, normal saline, by centrifugation at 800 RPM. The cells were resuspended in 0.2 ml of 1% methyl cellulose, smears were made and fixed for several hours in 95% v/v ethanol, then washed for 30 min in running, cold tap water. The smears were dipped in K5 nuclear emulsion, obtained from Ilfords Ltd., Essex, England, exposed for 10 to 14 days, and then developed and stained using Giemsa stain diluted 1/10 in buffered distilled water, prepared by dissolving a Gurr's buffer tablet (George T. Gurr Ltd., London), at pH 6.8, in 100 ml of water. To estimate the per cent of cells labeled, 1000 consecutive cells were counted on each slide.

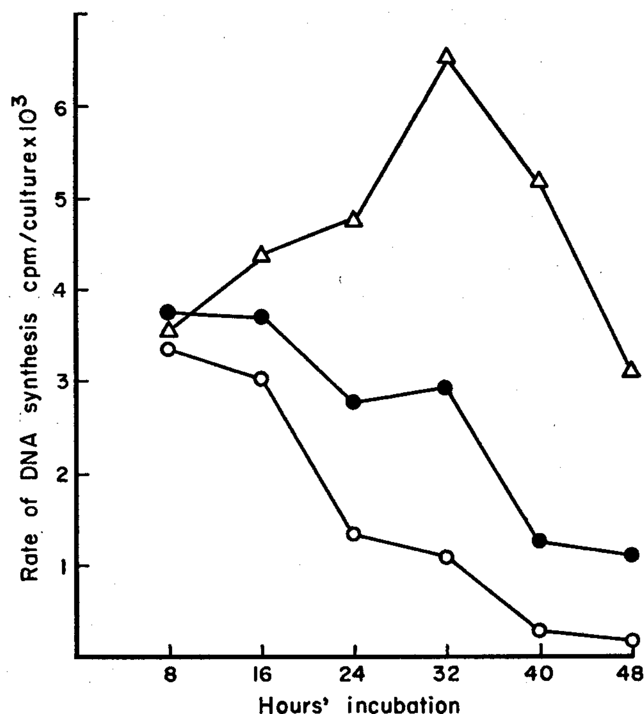
Irradiation of Cells.—Cultures were irradiated with a Marconi industrial model X-ray set, running at 250 kv and 15 ma to give a dose rate of 200 rad per minute using filters of 0.5 mm copper and 1.0 mm aluminum.

Estimation of Antibody-Producing Cells.—This was done according to the original method described (13, 14) and it should be pointed out that only cells producing γM-antibody were estimated. Medium was renewed every 3rd day by removing and replacing 1 ml without disturbing the cells. No additional SRC's or PHA was added after the cultures were set up. Suspensions of 10^7 cells were incubated in 1.5 ml of Eagle's minimum essential medium containing 15% pooled rabbit serum. No difference was noted if medium 199 (Morton, Morgan, and Parker) was substituted or hydrocortisone added.

RESULTS

The Effect of PHA on Deoxyribonucleic Acid (DNA) Synthesis by Rabbit Spleen Cells

The Stimulation of DNA Synthesis.—The effect of PHA on the rate of DNA synthesis in spleen cells from an immunized rabbit is shown in Text-fig. 1. Without antigen or PHA there was a rapid decline in the rate of DNA synthesis



TEXT-FIG. 1. The rate of incorporation of thymidine by rabbit spleen cells. ○, control; ●, antigen; and △, PHA. The rate of thymidine incorporation was measured by the CPM/culture in 8-hr incubation periods.

When antigen was added at the start of the incubation, a significant difference as compared to controls, in the rate of DNA synthesis, was noted during the 24- to 48-hr period of culture. PHA produced a more marked stimulation which reached a peak by 32 hr of culture. In contrast to soluble protein antigens which produced erratic responses in different experiments, PHA was constant in its stimulatory effect, reaching a maximum during the 30- to 40-hr period of incubation, although the degree of stimulation varied from experiment to experiment. The time of maximal response to PHA by spleen cells from a nonimmunized

rabbit was similar to that by spleen cells from an immunized animals, the peak of response being reached by 30 hr of culture.

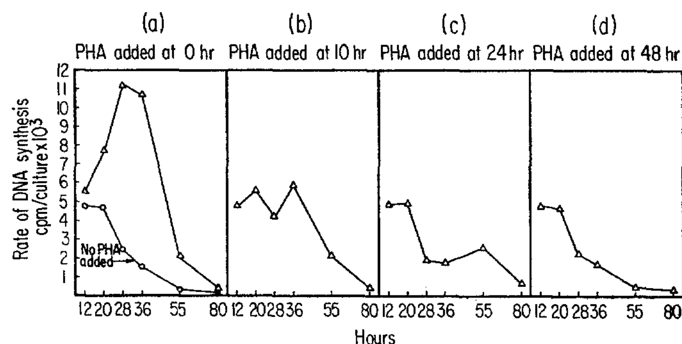
The results of many experiments indicated that the responses to PHA by spleen cells from animals killed after a booster injection of antigen were always greater than responses by spleen cells from nonimmunized rabbits.

TABLE I

The Relation between Concentration of Phytohemagglutinin and Stimulation of DNA Synthesis

PHA	Rate of DNA synthesis
μg/culture	*CPM/culture*
0	1,240
10	35,016
100	16,103
1,000	2,273

1.0×10^7 rabbit spleen cells were incubated in PHA, which was added initially. The rate of DNA synthesis was measured by the incorporation of thymidine-C^{14} during the 24- to 48-hr period of culture.



TEXT-FIG. 2. The effect of delay in adding PHA to cell suspensions. The rate of DNA synthesis was measured by the CPM/culture of thymidine-C^{14} incorporated during 8-hr incubation periods.

The Effect of Concentration of PHA on the Rate of DNA Synthesis.—The results in Table I show that the stimulatory effect of PHA on the rate of DNA synthesis in spleen cells measured during 24 to 48 hr of incubation was optimal at a concentration of PHA in the medium of 10 μg per culture. With amounts above those recorded, inhibition below control counts was noted and this was associated with morphological evidence of cell damage. In all results that follow, PHA was used at 10 μg per culture, as this concentration produced the greatest response in every experiment.

The Effect of Delay in Adding PHA on DNA Synthesis.—As shown in Text-

fig. 2, delay in adding PHA resulted in reduced response of the rate of DNA synthesis until, when added at 48 hr, no observable effect was noted in cells incubated for a further 36 hr. Incorporation of thymidine by cells incubated in PHA added at 0 hr, had virtually ended by 80 hr of culture.

Transfer Experiments

The Ability of Spleen Cells Incubated in Antigen or PHA to Stimulate DNA Synthesis in Spleen Cells Not Directly Exposed to these Materials.—Previous work (12) has shown that when spleen cells were primed by incubation in heterologous

TABLE II

Transfer Experiment

Combination of spleen cells	Rate of DNA synthesis in response to number of R2 cells added to 10^7 R1 cells			
	CPM/*culture*	CPM/*culture*	CPM/*culture*	CPM/*culture*
No. of R2 cells added to R1 cells	10^7	10^6	10^5	0
R1 + CR2 (160)	4,715	6,155	4,440	4,156
R1 + HSAR2 (183)	6,213	6,350	4,223	—
R1 + PHAR2 (1,626)	19,700	15,590	4,695	—

Rabbit R1 was immunized to HSA. Spleen cell suspensions of R2, nonimmunized, were incubated in medium alone, HSA at 1 mg/ml or PHA at 10 μg/ml for several hours, washed, and irradiated by a dose of 1000 rad (CR2, HSAR2, and PHAR2 respectively). The figures in brackets denote the rate of DNA synthesis of 10^7 such treated cells during their 24- to 48-hr period of culture. The rate of DNA synthesis by spleen cells from R1 was measured by the incorporation of thymidine-C^{14} during the 24- to 48-hr period of culture, after the addition of R2 cells, as CPM/culture

proteins for several hours, then washed and irradiated to reduce DNA synthesis, they were able to stimulate the rate of DNA synthesis in spleen cells from rabbits previously immunized to the same antigen. This response was antigen-specific. As shown in Table II similar experiments have been carried out with PHA.

When spleen cells from a nonimmunized rabbit were incubated in PHA for several hours, then washed, and irradiated to reduce DNA synthesis (PHAR2 cells) they were able to stimulate cells from another rabbit which had not been exposed directly to PHA (R1 cells). Since the PHAR2 cells were irradiated, the majority of counts in the combined cultures came from the nonirradiated R1 cells, and it can be seen that 10^6 PHAR2 cells were able to produce a significant stimulation of the rate of DNA synthesis in R1 cells.

The results also show that 10^7 R2 cells which have been incubated in HSA,

then washed and irradiated (HSAR2) were required to produce an antigen-specific stimulation of the rate of DNA synthesis in R1 cells. As the cells most effective in the transfer of stimulation due to antigen have been found to be macrophages (12) which stick to the glass incubation beaker and were left behind when the HSAR2 cells were transferred to beakers containing the responding R1 cells, this was considered to be the most likely explanation for the need for such a high number of HSAR2 cells. When 10^6 R2 cells were added to the cells from the immunized animals, stimulation occurred even when the added

TABLE III

The Effect of Separating Cells by a Millipore Membrane

Millipore chambers		Rate of DNA synthesis in cells outside chamber
Inside	Outside	
		CPM/culture
—	C	106
PHAR	C	126
—	C + PHAR	1066
—	PHAR	60

Suspensions of 1.1×10^7 spleen cells were incubated in PHA (10 μg/culture) for several hours, then washed, and irradiated with a dose of 1000 rad (PHAR). Control cells (C) and PHAR cells were incubated together or separated by a Millipore membrane to prevent direct cell contact. The rate of DNA synthesis was measured by the incorporation of thymidine-C^{14} during the 24- to 48-hr period of culture, as CPM/culture.

cells had not been preincubated in HSA or PHA (CR2 cells). This was a manifestation of the mixed cell reation (15, 16).

The Effect of Separation by a Millipore Membrane.—The results in Table III show that when spleen cells were primed by incubation in PHA for several hours, then washed, irradiated (PHAR), and separated from control nonirradiated cells (C) by a Millipore membrane, no stimulation of the rate of DNA synthesis was elicited in the control cells as compared to when both C and PHAR cells were incubated together.

Comparison of the Ability of Peritoneal Macrophages Incubated in Antigen or PHA to Stimulate DNA Synthesis in Spleen Cells.—The results in Table IV illustrate that macrophages, previously incubated in PHA at 100 μg per ml did not stimulate the rate of DNA synthesis in spleen cells. In contrast, macrophages incubated in HGG-stimulated DNA synthesis in spleen cells which were obtained from a rabbit previously immunized to that antigen. Inhibitory effects on the incorporation of thymidine-C^{14} were seen when high concentrations of

macrophages were employed. When rabbit red cells were incubated in PHA at varying concentrations, they also failed to transfer any stimulatory effect on the rate of DNA synthesis in spleen cells; these results have not been included in the table.

Radioautographic Studies

When suspensions of rabbit spleen cells were incubated in thymidine-H^3, only undifferentiated cells and cells with the characteristics of plasmablasts or

TABLE IV

Comparison of the Ability of Macrophages to Transfer Antigen (HGG) and PHA Stimulation of DNA Synthesis

Combination	Rate of DNA synthesis, CPM/culture				
	Macrophage No./culture			No macrophages added	
	6×10^6	6×10^5	6×10^4		
C + M (C)	1,995	2,330	3,825	Control	3,940
C + M (HGG)	2,400	3,056	8,546	HGG 1,000 μg/culture	17,330
C + M (PHA)	2,445	2,910	3,893	PHA 10 μg/culture	42,953

Peritoneal macrophages from a nonimmunized rabbit were incubated in medium alone, M (C), HGG at 1 mg/ml, M (HGG), and PHA at 0.1 mg/ml M (PHA) for several hours, washed, and 10^7 spleen cells from a rabbit immunized to HGG were added. The rate of DNA synthesis was measured by the incorporation of thymidine-C^{14} during the 24- to 48-hr period after adding the spleen cells to the macrophages. The results in the far right-hand column show the effects on DNA synthesis of incubating the spleen cells in medium alone, HGG or PHA.

lymphoblasts were labeled. No obvious morphological differences were noted in labeled cells obtained from cultures incubated in the presence or absence of PHA or specific antigens (Figs. 1 *a* and 1 *b*). No cells with the morphological appearances of macrophages or capable of sticking firmly to glass surfaces ever became labeled in freshly isolated cultures incubated in thymidine-H^3, but if such cultures were incubated for 48 hr or longer, occasional labeled macrophages were regularly seen.

In the experiments reported here (Table V), more than 2% of cells incorporated thymidine-H^3 in the 1st hr after isolation, when rabbits had received an intravenous booster injection of antigen 2 days before sacrifice (in some experiments as many as 10% were labeled), while about 1% or less of cells were labeled when animals were sacrificed without a booster injection.

When spleen cells from nonboosted animals were labeled as described in

TABLE V

Experiment	Booster	Labeled cells, per cent				Rate of DNA Synthesis
		1 hr		24 hr	48 hr	
						CPM/*culture*
R122 (HGG)	Yes	C	3	3	3	1,540
		HGG	3	3	6	4,910
		PHA	3	3	8	8,070
R119 (HGG)	Yes	C	1	1	0.6	1,330
		HGG	1	1	1.8	3,840
R114 (HGG)	Yes	C	3.6	3.0	2.6	1,175
		PHA	3.6	3.5	5.4	16,200
R147 (SRC)	Yes	C	2	2	2.4	710
		SRC	2	3.2	6.2	5,456
		PHA	2	3.4	6.5	5,850
		SRC + PHA	—	—	—	12,600
R155 (SRC)	Yes	C	2.8	3.0	3.0	376
		SRC	2.8	3.8	6.0	1,146
		PHA	2.8	3.6	5.2	1,620
		SRC + PHA	2.8	6.5	7.5	4,405
R146 (SRC)	No	C	0.6	0.6	0.5	330
		SRC	0.6	0.6	0.6	930
		PHA	0.6	0.6	0.6	1,671
		SRC + PHA	—	—	—	2,325
R148 (SRC)	No	C	1.4	1	1.6	253
		SRC	1.4	1	1.2	452
		SRC + PHA	—	—	—	6,980
R152 (SRC)	No	C	1.1	1.4	1.4	237
		SRC	1.1	1.0	1.0	833
		PHA	1.1	1.0	1.8	907
		SRC + PHA	1.1	1.0	1.8	1,470

Suspensions of spleen cells from rabbits immunized to HGG or SRC's were incubated in thymidine-H^3 for 1 hr, then washed by centrifugation in fresh medium, and resuspended in medium containing 10 μg/ml unlabeled thymidine. Aliquot suspensions of 1×10^7 cells were incubated in medium only (C), or in the presence of antigen (HGG or SRC), PHA or both (HGG + PHA or SRC + PHA). Cultures were harvested and radioautographs were prepared to determine the proportion of labeled cells. The rate of DNA synthesis in cultures was also measured during 24 to 48 hr, by the incorporation of thymidine-C^{14}.

Table V and then incubated in the presence of antigen or PHA, no significant change in the proportion of labeled cells was seen as compared to control cells incubated without these materials.

In contrast there was an increase in the proportion of labeled cells as compared to controls in cell suspensions from rabbits which had received a booster injection, indicating that cells stimulated to proliferate by the in vivo injection of antigen, divided in culture in the presence of PHA or antigen. A feature to

TABLE VI

	PFC's/10^6 spleen cells, days										Rate of DNA synthesis CPM/culture 24 to 48 hr
	0	1	2	3*	4	5	6	7*	8	9	
Controls	3,440	700	300	—	355	257	226	300	—	—	376
SRC	—	3,600	3,150	—	3,550	4,170	3,170	3,250	4,040	5,000	1,146
PHA	—	815	257	—	98	149	147	224	375	480	1,620
SRC + PHA	—	1,700	—	—	3,800	3,880	—	4,200	—	—	4,405
Controls	11,900	250	228	187	99	55	—	55	48	34	786
SRC	—	1,233	1,193	1,146	1,515	199	—	156	108	61	2,560
PHA	—	399	164	237	142	83	—	81	41	32	6,120
SRC + PHA	—	1,451	1,895	1,510	1,106	183	—	107	133	43	10,195

Suspensions of 10^7 spleen cells from 2 rabbits immunized to SRC's and given a booster injection 2 days before killing were incubated in the absence or presence of SRC's, PHA, or with both together. The proportion of plaque-forming cells/10^6 cells was estimated in individual cultures, and the rate of DNA synthesis was measured by the incorporation of thymidine-C^{14} during 24 to 48 hr of culture, as CPM/culture.

* Indicates change of medium.

be noted was that the stimulus provided by PHA and antigen together was greater than either separately.

It should be pointed out that the number of cells labeled at 48 hr represented only part of the population of cells responding in the cultures and the whole DNA counts indicate the rate of synthesis of DNA in all the responding cells during 24 to 48 hr.

Radioautographs using pulse labels at intervals after adding PHA or antigen indicated that, in nonboosted spleens, the in vitro cell response was from a population of cells which was not synthesizing DNA before receiving the mitogenic stimulus.

Hemolytic Plaque Formation.—Using the hemolytic plaque technique (15, 16), the proportion of specific antibody-forming cells (PFC's) in suspensions of spleens from rabbits immunized to whole SRC's was studied. The proportion of active cells was much higher in boosted than nonboosted spleens, but SRC's were required to be present in the medium for the maintenance of a plaque-forming population greater than that of controls cultured in their absence (Table VI). The number of PFC's was roughly proportional to the degree of stimulation of DNA synthesis by SRC's in these cultures. The presence of PHA with or without sheep red cells did not influence this number, despite the ability of PHA as shown in the results above, to stimulate cell proliferation in these cultures, and the summative effect on the rate of DNA synthesis resulting from the presence of both PHA and SRC's in the medium.

DISCUSSION

The rate of DNA synthesis in suspensions of rabbit spleen cells was stimulated by PHA as well as by antigens such as heterologous proteins and SRC's. Previous immunization by PHA was unnecessary, but responses to antigen were only seen in the spleens from rabbits previously immunized to the antigens used. The peak of response to PHA was about 30 hr after adding PHA to the cultures and no difference in the time of this peak was noted between cells from nonimmunized rabbits and cells from animals responding to an in vivo booster injection of antigen given 2 days before sacrifice, although the response to PHA by cells from boosted rabbits was always greater than from nonboosted animals.

Studies of the responses of human (2, 17) and rabbit (11, 5) peripheral white blood cells to PHA have shown that the peak response was much later, between 72 to 96 hr of culture. Delay in adding PHA to spleen cell suspensions resulted in reduced responses until no obvious stimulation was obtained when it was added at 48 hr after the initiation of the cultures. Delay in adding antigens to cell suspensions also resulted in reduced responses in the rate of DNA synthesis, and no stimulation during the 24- to 48- hr period of culture was produced by the addition of antigen 12 hr after the initiation of cultures (18). Therefore the stimulation of DNA synthesis in spleen cells in response to these materials was rapidly lost after isolation in cell suspension.

While antigens were always stimulatory in concentrations up to 10 mg/ml in the culture medium, the optimal concentration of PHA in all experiments was 10 μg/ml. Concentrations above this were less stimulatory and very high levels were found to be toxic.

The experiments reported here indicate that lymphocytes were able to take up the mitogenic factor in PHA and to transfer its stimulatory effect to responsive cells. The Millipore chamber experiment would suggest either that direct cell contact was required for this transfer to occur, or that the mitogenic material was attached to structures from cells which were too large to pass through the membrane of the chambers. In contrast to the results with antigens, perito-

neal exudate cells, which were incubated in PHA, were not able to stimulate DNA synthesis in spleen cells, indicating that even if these cells did take up the mitogenic material it was not available to stimulate the recipient cultures. Similar experiments with peripheral granulocytes and red cells, not reported here, were also negative and this is supported by the observation that absorption of PHA by red cells failed to remove its mitogenic activity (19). It would therefore appear that the mitogenic material in PHA was specifically taken up by lymphocytes alone and this is supported by studies with fluorescent-labeled PHA (20) which demonstrated that only lymphocytes from human peripheral blood became labeled and nuclear localization of fluorescent material was described in cells undergoing transformation into "blast" cells.

The radioautographic studies showed that cells stimulated to proliferation by an in vivo injection of antigen, responded in culture to the in vitro addition of PHA. The summative effect with antigen and PHA is of interest, and would suggest the possibility that different populations were being stimulated. Whether there was competition between antigen and PHA for stimulation of the responding population is hard to say. In experiments using spleens from rabbits which had received a course of immunization by antigens, but had not been stimulated for several months and were sacrificed without a booster injection, the same summative effect with a combination of antigen and PHA was seen although the population of cells responding was not actively dividing before contact with the two materials. A recent study (21) has shown that DNA synthesis in antibody-forming cells began 5 hr *after* injection of antigen and this would support the present results.

The studies using the plaque-forming technique to count the cells synthesizing specific antibody would support the idea that two separate populations were being stimulated, as no increase of plaque-forming cells occurred when PHA as well as SRC's was present in the medium, and with PHA alone the number of plaque-forming cells was not significantly different from control suspensions incubated in medium alone.

Although it has been claimed that human peripheral cells stimulated to growth by PHA were capable of γ-globulin synthesis (8, 9) this has not been substantiated by studies with rabbit peripheral cells (11). The present investigations indicate that part of a population of cells stimulated by the in vivo injection of antigen, responded to in vitro contact with PHA by continued cell division, but there was no evidence of stimulation of specific antibody production, while antigen stimulated both cell division and the maintenance of a specific antibody–forming population.

It has still to be shown that specific PFC's arise from cells stimulated to divide after in vitro contact with SRC's. In the absence of antigen in the medium, the number of antibody-producing cells fell during the first 48 hr and remained at about 10% of the initial levels for many days. When SRC's were added to the medium the number of PFC's was always greater than in controls

and in some experiments tended to rise, although no significant peak was seen as compared to the effect produced by SRC's added to spleen cell suspensions from previously immunized rabbits but sacrificed without a booster injection (22), the peak of response occurring on day 5 of culture.

The presence of SRC's in the medium might have maintained the production of antibody in cells stimulated by the in vivo booster injections, but it was not considered likely that the same cells continued to secrete antibody for so long in culture, as they were probably short-lived end cells (23).

However, the possibility that a stable messenger RNA is concerned in antibody synthesis must be seriously considered. Recent studies with *Bacillus megaterium* (24) have shown that the decay of messenger RNA can be reduced by energy starvation of the cells produced by anaerobiosis or by treatment with chloramphenicol. It was suggested that protection of messenger RNA could occur by virtue of being held on to ribosomes and that release from protection occurred when messenger was detached from the ribosomes.

Preliminary experiments with actinomycin D would suggest that the presence of SRC's in the culture medium of spleen cell suspensions from rabbits killed after a booster injection of SRC's resulted in the maintenance of specific PFC's, indicating retention of antibody synthesis and thus of maintenance of template RNA activity in the presence of a block to new RNA synthesis. These results are preliminary and require further study.

Since studies of the cellular events leading to a secondary immune response have shown that cell proliferation was necessary (23), it was likely that new cells were being stimulated to produce antibody by in vitro contact with antigen. Calculations, from the radioautographic studies, showed that the total number of DNA-synthesizing cells present in the spleen cell suspensions was sufficient to maintain the number of PFC's for more than 21 days. It would appear that a state of balance was achieved in the cell suspensions incubated with antigen, with a continuous replacement of cells actively producing antibody, but further analysis is required to assess whether antigen can produce a prolongation of antibody synthesis in individual cells.

Studies combining radioautography with the plaque-forming technique are in progress in an attempt to correlate the stimulation of DNA synthesis with the production of PFC's in these cultures. The present results would indicate that PHA, in vitro, stimulated the growth of cells which were already responding to in vivo contact with antigen but these cells did not produce antibody to the original antigen. The specific antigen stimulated only part of the responding population in vitro, with the maintenance of the antibody-synthesizing population in the cell suspensions. There was no evidence that PHA altered the response to antigen when added at the same time and this would suggest that they were acting independently on separate cell populations. The differences between these cell populations are of interest in view of the fact that they were originally stimulated to proliferate after in vivo contact with antigen. While it

would be rather premature to speculate on these results in relation to theories of antibody formation they would suggest that not all cells dividing in response to antigen were fated to synthesize specific antibody.

SUMMARY

Phytohemagglutinin (PHA) stimulated the rate of DNA synthesis in rabbit spleen cell suspensions. Unlike antigens, previous immunization to PHA was not necessary and the specific response could not be transferred by macrophages, although lymphocytes primed by incubation in PHA were able to stimulate other spleen cells not directly exposed to PHA. When rabbits were stimulated by in vivo immunization with antigens, spleen cells proliferating in response to antigen were stimulated to divide by in vitro contact with PHA.

Using the technique of specific hemolytic plaque formation by individual cells synthesizing γM-antibody to sheep red cells (plaque-forming cells), no evidence was obtained that stimulation of cell division by PHA resulted in specific antibody formation, although the presence of antigen resulted both in stimulation of cell proliferation and the production of plaque-forming cells. The presence of both sheep red cells and PHA in the medium of the same cell suspensions did not enhance the production of plaque-forming cells although there was a summative effect on DNA synthesis.

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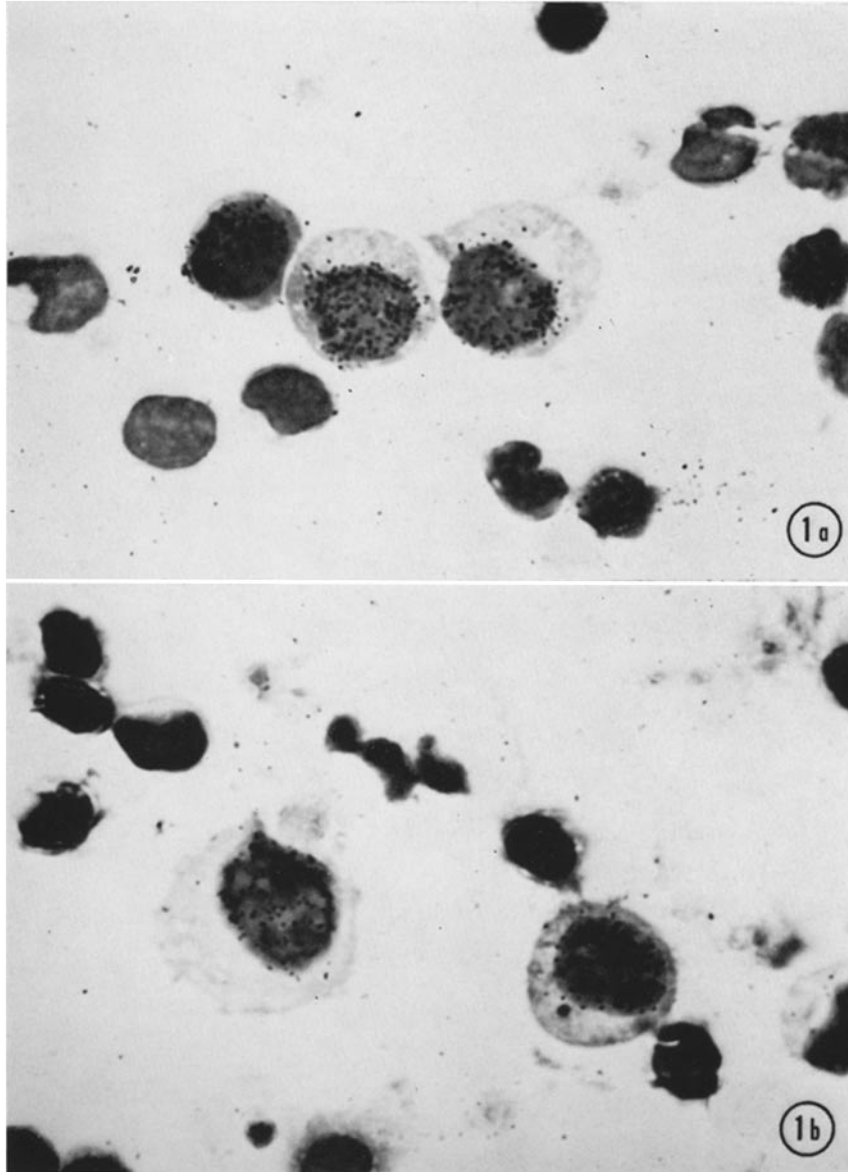
BIBLIOGRAPHY

1. Nowell, P. C., Phytohemagglutinin: an initiator of mitosis in cultures of normal human leukocytes, *Cancer Research*, 1960, **20**, 462.
2. Mackinney, A. A., Stohlman, F., and Brecher, G., The kinetics of cell proliferation in cultures of human blood, *Blood*, 1962, **19**, 349.
3. Tanaka, Y., Epstein, L. B., Brecher, G., and Stohlman, F., Transformation of lymphocytes in culture of human peripheral blood, *Blood*, 1963, **22**, 614.
4. Elves, M. W., Gough, J., Chapman, J. A., and Israels, M. C. G., Electron microscopy studies of lymphocytes, *Lancet*, 1964, **1**, 306.
5. Sabesin, S. M., Maglio, M. T., and Isselbacher, K. J., Effect of phytohaemagglutinin on rabbit peripheral lymphocytes, *Fed. Proc.*, 1965, **24**, 304.
6. Dutton, R. W., and Eady, J. D., An in vitro system for the study of the mechanism of antigenic stimulation in the secondary response, *Immunology*, 1964, **8**, 40.
7. Pearmain, G., Lycette, R. R., and Fitzgerald, P. H., Tuberculin-induced mitosis in peripheral blood leucocytes, *Lancet*, 1963, **1**, 637.
8. Bach, F., and Hirschhorn, K., γ-globulin production by human lymphocytes in vitro, *Exp. Cell Research*, 1963, **32**, 592.
9. Elves, M. W., Roath, S., Taylor, G., and Israels, M. C. G., The in vitro production of antibody by lymphocytes, *Lancet*, 1963, **1**, 1292.
10. Tao, T. W., Phytohaemagglutinin elicitation of specific anamnestic immune response in vitro, *Science*, 1964, **146**, 247.

11. Sell, S., Rowe, D. S., and Gell, P. G. H., Studies on rabbit lymphocytes in vitro. III. Protein, RNA, and DNA synthesis by lymphocyte culture after stimulation with phytohaemagglutinin, with staphylococcal filtrate, with anti-allotype serum, and with heterologous antiserum to rabbit whole serum, *J. Exp. Med.*, 1965, **122,** 823.
12. Harris, G., Studies of the mechanism of antigen stimulation of DNA synthesis in rabbit spleen cultures, *Immunology*, 1965, **9,** 529.
13. Jerne, N. K., and Nordin, A. A., Plaque formation in agar by single antibody producing cells, *Science*, 1963, **140,** 465.
14. Jerne, N. K., Nordin, A. A., and Henry, C., The agar plaque technique for recognizing antibody-producing cells, *in* Cell-Bound Antibodies, (B. Amos and H. Koprowski, editors), Philadelphia, Wistar Institute Press, 1963, 109.
15. Bain, B., Vas, M. R., and Lowenstein, L., The development of large immature mononuclear cells in mixed leukocyte cultures, *Blood* 1964, **23,** 108.
16. Chapman, N. D., and Dutton, R. W., The stimulation of DNA synthesis in cultures of rabbit lymph node and spleen cell suspensions by homologous cells, *J. Exp. Med.*, 1965, **121,** 85.
17. Cooper, E. H., Barkhan, P., and Hale, A. J., Observations on the proliferation of human leukocytes cultured with phytohaemagglutinin, *Brit. J. Haematol.*, 1963, **9,** 101.
18. Harris, G., unpublished observations.
19. Kolodny, R. L., and Hirschhorn, K., Properties of phytohaemagglutinin, *Nature*, 1964, **201,** 715.
20. Michalowski, A., Jasinska, J., Bryosko, W. J., and Nowoslawski, A. Cellular localization of the mitogenic principle of phytohaemagglutinin in leucocyte cultures, *Exp. Cell Research*, 1964, **34,** 417.
21. Cohen, E. P., and Talmage, D. W., Onset and duration of DNA synthesis in antibody forming cells after antigen, *J. Exp. Med.*, 1965, **121,** 125.
22. Richardson, M., and Dutton, R. W., Antibody synthesising cells: Appearance after secondary antigenic stimulation in vitro, *Science*, 1964, **145,** 655.
23. Nossal, G. J. V., and Mäkelä, O., Autoradiographic studies on the immune response. I. The kinetics of plasma cell proliferation, *J. Exp. Med.*, 1962, **115,** 209.
24. Fan, D. P., Higa, A., and Levinthal, C., Messenger RNA decay and protection, *J. Mol. Biol.*, 1964, **8,** 210.

EXPLANATION OF PLATE 56

FIGS. 1 *a* and 1 *b*. Radioautographs of cells synthesizing DNA: Fig. 1 *a*, in the presence of antigen; Fig. 1 *b*, in the presence of PHA. Thymidine-H^3 was added for 1 hr at the peak of response which occurred at 30 hr of incubation. × 1220.



(Harris and Littleton: Rabbit spleen cell suspensions)