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THE MITOGENIC RESPONSE OF HUMAN B LYMPHOCYTES TO PHYTOHAEMAGGLUTININ

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SUMMARY

Further evidence is given here of a mitogenic response to phytohaemagglutinin by human B lymphocytes. Immunofluorescent staining of unfractionated lymphocytes, and thymidine uptake by column purified populations of B and T cells, indicates that the dose–time response of these two populations is comparable. PHA transformation of B lymphocytes appears to be reduced or absent in chronic lymphocytic leukaemia.

INTRODUCTION

Evidence has previously been presented suggesting that, in the human, bone marrow-derived (B) lymphocytes respond to the mitogenic stimulus of phytohaemaglutinin (PHA) (Phillips and Roitt, 1973). This is in contrast to the mouse and rat, where the PHA responding cells are considered to be specifically thymus-derived (Doenhoff *et al.*, 1970; Johnston & Wilson, 1970; Jones, 1972), although there are recent reports to the contrary (Vischer, 1972; Piguet & Vassalli, 1972). We present here further evidence of PHA-induced B-cell transformation in the human, and details of the kinetics of this response.

MATERIALS AND METHODS

Lymphocytes

Peripheral blood lymphocytes were separated from heparinized blood by dextran sedimentation (equal volumes of blood and 3·5% dextran in 0·9% saline, 30 min at 37°C). The leucocyte-rich supernatant was removed, centrifuged at 150 *g* for 10 min, and the cell pellet washed twice in Eagles' minimum essential medium (MEM). Tonsil lymphocytes were obtained by teasing the tissue in cold MEM, and allowing debris to settle. Polymorphs were removed by incubation with iron powder.

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Lymphocyte separation on immunoabsorbent columns

Lymphocyte populations enriched for non-immunoglobulin-bearing (T) or immunoglobulin-bearing (B) cells were prepared by fractionation on immunoabsorbent columns of Sephadex G-200 or Degalan beads, containing bound purified rabbit anti-human light chain, as previous described (Phillips and Roitt, 1973). Alternatively, B cells were obtained by fractionation on an immunoabsorbent column consisting of sheep anti-human Fab bound to Sephadex G-200 by means of a spacer molecule of gelatin. Retained B cells were recovered from the column by collagenase digestion of the spacer molecule. Details of this procedure have been given elsewhere (Thomas & Phillips, 1974).

Cell culture

Lymphocytes were cultured with PHA (Wellcome Research Laboratories, reagent grade) in MEM supplemented with 10% pooled human serum ($1–2 \times 10^6$ cells/ml, 95% air, 5% CO_2). Bacterial contamination of tonsil lymphocytes was controlled by washing the cells three times in sterile medium before culturing with added gentamicin (Roussell) and vancomycin (Lilly) (25 μg/ml). Macro cultures were set up in 10 or 20 ml culture flasks, and micro cultures in plastic microtest tissue culture plates (Falcon Plastics) 0·1 ml per well, maintained in a humid atmosphere.

Incorporation of thymidine

Cultures were incubated with [^{14}C]thymidine or [^{3}H]thymidine (0·25 μCi/ml) for 12 hr before collection. Cells were washed with MEM and the DNA precipitated with 5% trichloracetic acid as previously described. Precipitates were counted in a liquid scintillation counter, using a toluene-based scintillant. For autoradiography, cytosmears were prepared, and fixed with methanol. Slides were dipped in Ilford K5 nuclear emulsion, and exposed at 4°C for 4–7 days. After development (Kodak D19b, 5 minutes, 16°C) they were stained with May–Grünwald–Giemsa, and examined under a light microscope.

Antisera

All antisera, with the exception of rabbit anti-human light chain (ALC), were rendered specific by absorption with the appropriate insolubilized globulins. They were used routinely at a dilution of 1/16.

Immunofluorescent staining

Viable lymphocytes were incubated with rabbit antisera or normal rabbit serum (5×10^6 cells, 0·1 ml antiserum) for 30 min at 0–4°C, washed three times with cold MEM and incubated for 30 min with goat anti-rabbit fluorescein conjugate. After a further three washes in MEM, cells were examined with a Leitz ortholux microscope under ultraviolet light. Blast cells were identified by viewing the same field under phase-contrast. In some instances, immunofluorescent autoradiographs were prepared. In this case, to remove fluorescent debris, lymphocytes were washed finally in 7% BSA. They were then cytosmeared, fixed in ethanol, and autoradiographs prepared as previously described. After development these preparations were not stained with May–Grünwald–Giemsa but examined under ultraviolet light and phase-contrast.

RESULTS

Thymidine-labelled immunoglobulin-bearing blasts

Tonsil lymphocytes from three donors were cultured with PHA, harvested at 72 hr, and stained in suspension with ALC. Autoradiographs were prepared from these stained cells. The percentage of [^{3}H]-labelled blasts positive for ALC was approximately the same in stimulated and unstimulated cultures (Table 1).

TABLE 1. Percentage of thymidine-labelled lymphoblasts positive for ALC in PHA cultures at 72 hr

Donor		[^{3}H]Thymidine-labelled (%)†	[^{3}H]Thymidine-labelled‡ ALC-positive (%)
E (34) *	PHA	13	25
	No PHA	1	n.d.§
F (33)	PHA	17	25
	No PHA	2	14
G (41)	PHA	21	22
	No PHA	9	20

* Numbers in parentheses refer to ALC-positive lymphocytes at zero time (%).

† Autoradiographs were prepared from lymphocytes stained in suspension with May–Grünwald–Giemsa.

‡ Autoradiographs were prepared from lymphocytes stained in suspension, and viewed under fluorescence/phase-contrast.

§ N.d. = not determined.

Aliquots stained with NRS were always $< 5\%$ positive.

Surface staining of lymphoblasts

Tonsil lymphocytes from four donors were stained in suspension with antisera to ALC, IgM, IgA, IgG and κ and λ light chains, before and after culturing with PHA (Table 2). In each case, when the total percentage of lymphocytes (zero time) or blasts (at 72 hr) which were positive for IgG, IgA, IgM was summated, the discrepancy between the total, and the value obtained with ALC serum was $\pm 10\%$. Similar results were obtained with antisera to κ and λ light chain determinants.

There was no significant change in the relative percentages of cells expressing different immunoglobulin class determinants, or light chain determinants after culturing with PHA (Table 3).

Kinetics of mitogenic response: mixed cell population

Fig. 1 shows the time–dose response curves for B cell transformation in a mixed cell population. Tonsil lymphocytes were cultured with PHA at dilutions varying from 1/25–1/1600 and aliquots harvested at 48, 72 and 96 hr. In the absence of PHA, the percentage of ALC-positive lymphocytes remained approximately constant throughout the culture period. At the highest concentration (1/25) PHA seemed to be selectively toxic for B cells. At 48 or 72 hr both total blast transformation and the percentage of ALC-positive blasts

TABLE 2. Percentage of lymphocytes stained with antisera to immunoglobulin classes, and light chain determinants, before and after culturing with PHA

Donor		ALC*	IgG	IgA	IgM	Total immuno-globulin†	κ	λ	Total light-chains‡
A	Preculture lymphocytes	60	31	7	13	51	46	12	58
	Post culture blasts	27	18	5	12	35	20	10	30
B	Preculture lymphocytes	34	27	3	8	38	14	22	36
	Post culture blasts	15	9	1	1	11	7	4	11
C	Preculture lymphocytes	33	22	6	12	40	24	10	34
	Post culture blasts	25	17	1	6	24	24	5	29
D	Preculture lymphocytes	41	36	2	3	41	11	19	30
	Post culture blasts	21	17	1	1	19	28	1	29

* ALC refers to antiserum raised against both κ and λ light chain determinants.

† Total immunoglobulin is derived by summating percentages obtained with antisera to immunoglobulin classes.

‡ Total light chains were derived by summating percentages obtained with anti-κ and anti-λ antiserum.

Staining with normal rabbit serum was always $<3\%$.

TABLE 3. Relative percentages of cells expressing immunoglobulin class and light chain determinants prior to culture (PC) and post culture (PoC) with PHA, 72 hr

Donor		IgG	IgA	IgM	κ	λ
A	PC	60	13	27	79	21
	PoC	52	14	34	66	33
B	PC	71	8	21	61	38
	PoC	81	9	9	63	37
C	PC	55	15	30	70	29
	PoC	70	4	25	82	18
D	PC	88	6	6	63	36
	PoC	89	6	5	96	3

Values were calculated from Table 2.

were optimum at a PHA dilution of 1/100. However, on day 4, the percentage of total lymphoblasts was optimum at 1/400, whereas the percentage of ALC-positive blasts was at a minimum. Similar results were obtained with tonsil lymphocytes from two other donors.

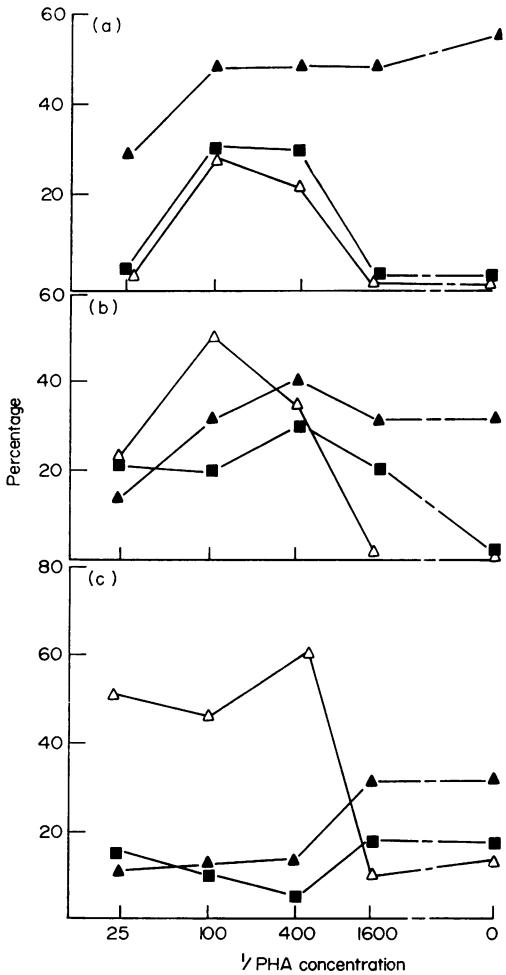

FIG. 1. Dose–response curves for B-cell transformation in a mixed cell population at different times. (a) 48 hr. (b) 72 hr. (c) 96 hr. (▲) Total ALC-positive cells. (■) Percentage of blasts positive for ALC. (△) [^{3}H]Thymidine-labelled blasts.

Kinetics of the mitogenic response: column fractionated cells

Tonsil lymphocytes enriched for T or B cells were prepared on Sephadex–ALC columns, and cultured in microculture plates with PHA at dilutions varying from 1/25 to 1/1600. Cultures were harvested at 48, 72, and 96 hr and ^{14}C incorporation measured by TCA precipitation. In Fig. 2, the dose–response curves for T, B and unfractionated lymphocytes

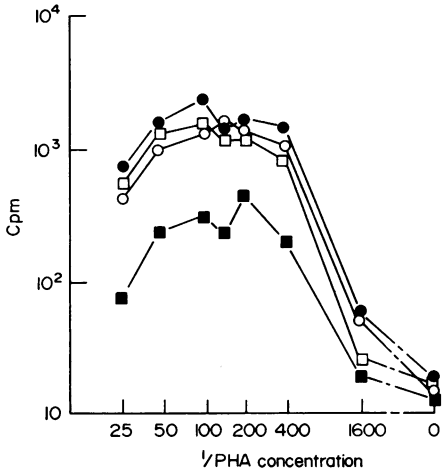

FIG. 2. Dose–response curves at 72 hr for T, B and unfractionated lymphocytes compared with that obtained with lymphocytes passed through a Sephadex column containing bound normal rabbit immunoglobulin. Each point is an average of five cultures. (●) T cells, <1% ALC-positive. (■) B cells, 85% ALC-positive. (○) Unfractionated cells, 55% ALC-positive. (□) Cells passed through normal rabbit immunoglobulin column, 50% ALC-positive.

are compared with that obtained with lymphocytes passed through a Sephadex column containing bound normal rabbit immunoglobulin (cultures harvested at 72 hr). The results obtained with the mixed cell population, and with lymphocytes passed through the control column are closely comparable. Time–dose response curves for T, B, or unfractionated lymphocytes are given in Fig. 3. In each case, plateau values were obtained with PHA concentrations of 1/100–1/400, and were maximal at 96 hr. The dose–response curve obtained

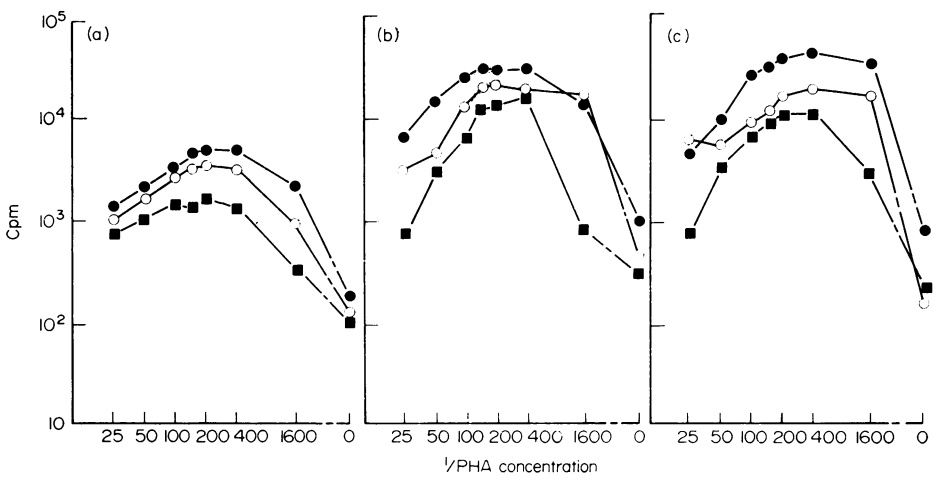

FIG. 3. Dose–response curves for T, B, or unfractionated lymphocytes at different times. Each point is an average of five cultures. (a) 48 hr. (b) 72 hr. (c) 96 hr. (●) T cells, 3% ALC-positive. (■) B cells, 90% ALC-positive. (○) Unfractionated cells, 30% ALC-positive.

with B cells was below that obtained with T cells, or unfractionated cells. Two further experiments gave similar results.

Table 4 shows the percentage of transformed cells in cultures of B, T and unfractionated cells, PHA dilution 1/100, at 72 hr. In this case, B cells were prepared on Sephadex–gelatin–anti-Fab columns, as contamination with residual Sephadex particles makes B-cell populations obtained from Sephadex–ALC columns unsuitable for autoradiography. T cells were fractionated on Degalan columns.

TABLE 4. PHA cultures of fractionated lymphocytes: percentage [^{3}H]thymidine-labelled at 72 hr

Donor		ALC positive (%): total cells			[^{3}H]Thymidine-labelled (%)		
		T *	B †	UF ‡	T	B	UF
4	Preculture	6	75	47			
	Postculture	2	77	50	26	21	30
5	Preculture	1	89	45			
	Postculture	0	80	44	55	34	30

* T = thymus-derived.
† B = bone marrow-derived.
‡ UF = unfractionated cells.

TABLE 5. ALC-positive blasts in PHA cultures of peripheral blood lymphocytes from patients with chronic lymphocytic leukaemia

Donor		ALC-positive blasts (%)	Blasts (%)
FT (68)		1	42
GS (75)		2	34
HR (63)		25	10
DS (70)		3	24
AS (43)	72 hr	1	21
	110 hr	3	51

* Numbers in parentheses signify the percentage of total lymphocytes ALC-positive prior to culture.

† The percentage of ALC-positive blasts in cultures of normal peripheral blood lymphocytes was 25 ± 5 (the mean value for fifteen donors).

‡ The percentage of blasts was determined by phase-contrast.

Staining with NRS was always $< 5\%$.

Chronic lymphocytic leukaemia

Peripheral blood lymphocytes from patients with chronic lymphocytic leukaemia were cultured with PHA (1/100). They were harvested at 72 hr (and in one case 110 hr) and the percentage of ALC-positive blasts determined by viable cell staining (Table 5). In only one case (HR) did there appear to be B-cell transformation.

Agammaglobulinaemia

Peripheral blood lymphocytes from one patient with congenital agammaglobulinaemia were cultured with PHA 1/100, 72 hr. There was no significant B-cell transformation (Table 6).

TABLE 6. ALC-positive blasts in PHA cultures (72 hr) of peripheral blood lymphocytes from patient with congenital agammaglobulinaemia

	ALC	IgM	IgG	[^{3}H]Thymidine-labelled (%)
Preculture	7	0	4	
Postculture	3	2	0	70

DISCUSSION

Autoradiography of cells incubated with [^{3}H]thymidine and stained in suspension indicates that in PHA cultures of human lymphocytes there are blast cells bearing surface immunoglobulin. It is unlikely that the immunoglobulin is passively absorbed, since staining with antisera to IgG, IgA and IgM suggests that these determinants are on separate cells. Similarly, as has also been shown by Biberfeld *et al.* (Biberfeld, Biberfeld & Perlmann, 1971), κ and λ light chain determinants appear to be expressed independently. Furthermore, there was no change in the relative percentages of cells expressing these determinants, after culturing with PHA. These results, together with the previous report showing that PHA cultures of lymphocyte populations enriched for T cells contain little or no immunoglobulin-bearing lymphocytes, strongly suggest that these are B lymphoblasts. Recently it has been shown that there are antigens specific for B or T lymphoblasts in the human. Antisera raised against either of these antigens react with a subpopulation of PHA-transformed human lymphoblasts (Thomas & Phillips, 1973).

The results obtained with both unfractionated and fractionated lymphocytes indicate that T and B lymphoblasts have similar time–dose response curves. The fall in the percentage of immunoglobulin-bearing blasts by 96 hr compared with the percentage of total blasts in the culture, might be due to a 'diluting out' effect by T lymphoblasts, or to differential viability or both. Since immunoglobulin-positive blasts were determined as a percentage of total blasts rather than total lymphocytes, this result cannot be conclusively explained here.

The results obtained with T and B lymphocytes fractionated on a Sephadex–ALC column suggest that the mitogenic response of the cells is considerably below that of T lymphocytes and unfractionated cells. However, this is probably a technical artifact, due to contamination of the cultures with Sephadex debris, since with an alternative method of fractionation on a Sephadex–gelatin–anti-Fab column, the percentage of [^{3}H]thymidine-labelled cells in cultures of T, B and unfractionated cells was closely comparable. These results have been confirmed elsewhere (Thomas & Phillips, 1974).

It has been reported that in chronic lymphocytic leukaemia the PHA response is reduced (Smith, Cowling & Barker, 1972) and an abnormally high percentage of the peripheral blood lymphocytes express surface immunoglobulin (Wilson & Nossal, 1971; Papamichael, Brown & Holborow, 1971). It is therefore of interest that in only one of five CLL patients tested here, was there a demonstrable B-cell response to PHA.

Ripps & Hirschorn (1967) found an increase in intracellular immunoglobulin in human lymphocytes cultured with PHA, confirming similar reports (Bach & Hirschhorn, 1963; Elves *et al.*, 1963). They found no such increase in patients with agammaglobulinaemia. In the one case of congenital agammaglobulinaemia tested here, although a high percentage of lymphocytes were transformed, only 3% expressed surface immunoglobulin. It would obviously be of interest to determine what, if any, B-cell transformation is induced in further cases of congenital agammaglobulinaemia, and also in acquired agammaglobulinaemia where the numbers of circulating B lymphocytes are normal (Cooper, Lawton & Bockman, 1971).

The results presented here, while demonstrating that there is a B-cell response in PHA-stimulated cultures of human lymphocytes do not indicate whether PHA stimulates B lymphocytes directly or, as has been suggested in the mouse (Piguet & Vassalli, 1972; Vischer, 1972), by means of a T-cell mitogenic factor. It is possible that a small percentage of the lymphocytes digested from the Sephadex columns are T cells, since only a maximum of 90% of these lymphocytes stain with ALC, although it has been shown that similar populations of mouse (Shlossman & Hudson, 1973) and chicken (Hudson, personal communication) lymphocytes recovered from these columns do not stain with antisera specific for T lymphocytes. This may be determined by testing purified populations with antisera to B- and T-lymphoblastoid antigens, after transformation with PHA.

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