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LYMPHOCYTE ACTIVATION

I. RESPONSE OF T AND B LYMPHOCYTES TO PHYTOMITOGENS

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SUMMARY

The selectivity of phytomitogens for T (thymus derived/dependent) and B ('bursa-equivalent' dependent/derived) lymphocytes from the mouse spleen has been investigated. Responses of normal spleen cell cultures were compared with those of cultures of selected B cells. The latter were obtained from three sources (1) spleen cells of mice that had been thymectomized as adults, lethally irradiated and reconstituted with syngeneic bone marrow cells pretreated with anti-θ serum (2) spleen cells from congenitally athymic ('nude') mice and (3) spleen cells from normal mice treated with anti-θ serum plus guinea-pig complement prior to culture.

Using a variety of different culture conditions it was shown that B cells respond well to pokeweed mitogen, and poorly if at all to phytohaemagglutinin. Responsiveness to the latter mitogen in normal spleen cell cultures appears to be a property of T cells.

INTRODUCTION

The mitogenic activity of plant lectins or phytohaemagglutinins has been extensively studied since its discovery by Nowell (1960) (reviewed by Naspitz & Richter, 1968). Although most attention has been directed towards the mitogen from *Phaseolus vulgaris* (phytohaemagglutinin or PHA), extracts from most plants of the Leguminosae family have similar properties. A considerable variety of other substances including bacterial products and antibodies activate lymphocytes in much the same fashion as phytomitogens (Ling, 1968; Oppenheim, 1968; Hirschhorn, 1970). Much of the impetus for this work derives from the observation that the gross morphological and biochemical characteristics of mitogen induced lymphocyte responses *in vitro* are very similar to antigen induced immune reactions *in vivo*. It has been suggested that the various mitogenic substances 'bypass' the requirement for antigenic recognition and induce cells to undergo that pattern of response 'normally' dependent upon

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strict immunological activation (Coulson & Chalmers, 1964). It is therefore generally considered that the lymphocyte activation phenomenon *in vitro* offers not only a means of analyzing the biochemical events involved in cellular de-repression (Pogo, Allfrey & Minsky, 1966; Hirschhorn *et al.*, 1969), but is also of considerable value as a clinical tool for monitoring the immunological competence of lymphocytes from both patients with various immunological disorders and those undergoing immunosuppressive therapy (Oppenheim, 1968).

It has become increasingly clear in recent years that lymphocytes are heterogeneous and that in essence a dichotomy exists between thymus-derived (T) cells and 'bursa-equivalent'-derived (B) cells (reviewed by Meuwissen, Stutman & Good, 1969; Roitt *et al.*, 1969; Davies *et al.*, 1971). It would therefore seem pertinent to inquire into selectivity, if any of various mitogens, towards T and B lymphocytes. This approach has already proved fruitful with Phytohaemagglutinin (PHA). Studies with cells from animals or human beings with selective immunological impairments, induced experimentally or occurring 'naturally', have clearly demonstrated that the responsiveness to PHA is predominantly, if not uniquely, a property of T lymphocytes (references in Table 5 in Discussion).

The phytomitogen Pokeweed (PWM) from *Phytolacca americana* has also been widely studied although to a lesser extent than PHA (Farnes *et al.*, 1964; Chessin *et al.*, 1966). One of the most interesting aspects of the response of lymphocytes to the former substance is that a considerable proportion of activated cells develop ultrastructural characteristics of the plasma cell line *in vivo* (Douglas *et al.*, 1967; Douglas & Fudenberg, 1969; Barker, Lutzner & Farnes, 1969). Since this pattern of response is a characteristic property of B lymphocytes we might therefore predict that, in contrast to PHA, PWM stimulates B cells *in vitro*. We have attempted to show that this is correct by analysing the response to PHA and PWM in mouse lymphocyte suspensions devoid of T cells. In addition to the conventional approach of thymectomy as a means of T deprivation we have taken advantage of the finding that antisera to the theta (θ) antigen in the presence of guinea-pig complement appears to be selectively toxic for T cells (Raff, 1969). Three types of T-deprived spleen cell suspensions have been studied: (1) cells from mice previously thymectomized as adults, lethally irradiated and subsequently reconstituted with syngeneic bone marrow cells which had been pretreated with anti-θ serum—referred to below as B spleen—(2) cells after direct treatment with anti-θ serum (plus complement) and (3) cells from a 'nude' strain of mouse with congenital thymic aplasia.

MATERIALS AND METHODS

Mice

Normal (control) mice. Six- to twelve-week-old inbred CBA ($H\text{-}2^k$) mice were used in all experiments.

T-cell deprived mice. Four-week-old CBA mice were thymectomized. Two weeks later they were lethally X-irradiated with 850 r and reconstituted i.v. 24 hr later with $5\text{–}10 \times 10^6$ viable bone marrow mononuclear cells which had been pretreated with anti-θ serum plus guinea-pig complement (under conditions which were optimal for lysing θ positive lymphocytes in spleen and lymph node). Mice were used as a source of spleen cells (B spleen cells) 3–8 weeks after reconstitution.

Congenitally athymic ('nude') mice (Flanagan, 1966; Pantelouris, 1968). These mice were

bred from a nucleus of mating pairs kindly provided by Dr D.S. Falconer of the Institute of Animal Genetics, Edinburgh. They were used at 4–8 weeks of age.

X-irradiation

Mice were X-irradiated with a cobalt-60 source at 68 rads/min, whole body irradiation, 190 cm from the source. They received a total of 850 r.

Lymphocyte cultures

1. *Cell sources and manipulations.* All techniques were carried on aseptically. Spleens were placed in a 90 mm Petri dish (Sterilin Ltd., Cat. No. N7) containing 5 ml cold Eagle's M.E.M. The contents of spleens were teased out with forceps. The cell suspension was pushed through a series of needles of graded sizes and transferred to a plastic tube (NUNC, Denmark). Any remaining cell aggregates were allowed to sediment for 5 min. The supernatant single cell suspension was transferred to a second tube and centrifuged 10 min at 140 *g* at 4°. The cells were resuspended in 5 ml medium and counted with Trypan blue. The suspension was adjusted to contain 2×10^6 living leucocytes per ml.

Some experiments involved additional treatment of cell suspensions prior to culture. To eliminate the vast majority of polymorphonuclear cells (in 'nude' mouse experiments) and macrophages (in a study of the stimulating capacity of foetal calf serum) or dead cells (after anti-θ serum treatment) the cell suspensions were filtered through cotton wool at 37°. Suspensions treated in this way contained less than 1% phagocytic cells (colloidal carbon uptake) with more than 95% viable cells.

2. *Culture media.* Two varieties of culture media have been used. (a) an Eagle's Minimal Essential Medium (M.E.M.) with double strength amino acids, supplemented with 10% heat inactivated foetal calf serum (FCS) (Rehautin, Armour Pharmaceutical Int., Kaukakee, Ill. USA), (b) RPMI-1640 medium (Flow Labs. Ltd.) supplemented with 10% heat inactivated foetal calf serum (Flow Labs. Ltd. Batch No. L40218). Both media were further supplemented with freshly prepared glutamine solution (400 mM/2 ml per 100 ml medium) and antibiotics (Penicillin 200 U/ml and Streptomycin 100 μg/ml).

3. *Cultivation conditions.* Three types of culture conditions have been used. In most of experiments the cultures were set up in screw cap Falcon tubes (12 × 75 mm, area of bottom approximately 120 mm^2, Gateway Int., Cat. No. 2003). 2×10^6 leucocytes were suspended in 1 ml total volume.

To determine the effect of cell density on lymphocyte stimulation a microplate method was used. The microplates (Cooke Ltd., Microtiter R) contained 96 wells with flat bottoms, each approximately 0·2 ml capacity with 26 mm^2 surface area. The examined cell density range spanned over $1 \times 10^5 - 1 \times 10^6$ leucocytes per well*. In the above two culture systems the medium consisted of 10% FCS (Rehautin) in Eagle's M.E.M.

The third type of culture system was a screw cap plastic vial with flat bottom (area 180 mm^2, 8 ml capacity, Sterilin Ltd., Cat. No. P. 118/s). 3×10^6 leucocytes were cultured in 1·5 ml RPMI-1640 with 10% FCS (Flow Labs.).

All cultures were bubbled with a gas mixture of 7% CO_2, 10% O_2 and 83% N_2, and placed into a pressure cooker containing a buffer solution. This vessel was put into thermostate of 37°. The 7% CO_2 tension was maintained during the period of cultivation.

All cultures were set up in duplicate except where stated.

* Lymphocytes were always plated and allowed to sediment before the stimulant was added.

4. *Measurement of responses.* In the majority of experiments both RNA and DNA synthesis were examined in the same cultures. Double labelling was performed by adding 0·017 μCi [^{14}C]uridine (57 mCi/mmol, Radiochemical Centre, Amersham) after 14 hr incubation and 0·1 μCi [^{3}H]thymidine (5Ci/mmol, Radiochemical Centre, Amersham) per 1 ml of culture fluid at 36th hr. The cultures were terminated after 60 hr. In microplate experiments [^{3}H]thymidine (0·3 μCi into each well) was added at 36 hr and the cultures harvested at the 60th hr.

After 60 hr cultivation the cell suspensions were centrifuged and the cells washed once in cold phosphate buffered saline (lacking calcium and magnesium ions). One drop 3% BSA as carrier protein was dropped onto the cell pellet and after adding 1·5 ml of cold 5% TCA the tubes were left for precipitation for at least 30 min. The precipitates were washed in 1·5 ml of cold 5% TCA followed by 2 ml cold methanol. The precipitates were washed onto membranes (GF/C 2·5 cm, Whatman) in a 'Manifold' multiple sample collector (Millipore). The membranes were left to dry and subsequently placed into scintillation vials. 0·3 ml solubilizer (Soluene TM 100, Packard) was added and after incubation in 37° overnight or at 55° for 2 hr, 5 ml scintillation fluid (5 g PPO and 0·1 g POPOP in 1 litre toluene) was put into each vial. These were kept at 4° overnight and counted in a Beckman LS-200B liquid scintillation system.

To study protein synthesis by [^{3}H]leucine uptake (L-leucine-4,5-H-3, 57 Ci/mmol, 10 μCi/ml of culture fluid, Radiochemical Centre, Amersham) the preparation of cells was essentially the same except that the use of carrier protein was omitted and after the third washing with TCA the precipitate was transferred to the membranes.

5. *Viability and cytologic examinations.* Dye exclusion tests (0·4% Trypan blue) were routinely performed on cultivated cell suspensions. Smears were regularly prepared and after fixing in methanol, stained with Giemsa.

Phytomitogens

1. *Phytohaemagglutinin (PHA).* Two different PHA preparations have been used in these experiments: (a) Wellcome PHA (PHA-W), reagent grade, lot K 2381, and (b) a purified preparation of PHA (p-PHA), kindly supplied by Dr S. Yachnin. This material gave a single band on polyacrylamide gel electrophoresis.

2. *Pokeweed mitogen (PWM).* This stimulant was purified from the plant stems (*Phytolacca americana*) using the method described by Boregson *et al.* (1966). Stimulants were stored at −20° in small aliquots.

Anti-θ serum and anti-mouse 'B' lymphocyte-antigen (MBLA) serum

These sera were a gift from Dr M. Raff. Details of their specificity and method of production here been published (Raff, 1969; Raff, Nase & Mitchison, 1971).

Anti-θ serum treatment of spleen cell suspensions

0·5 ml anti-θ serum was added to 1·5 normal spleen suspension containing 80×10^{6} living leucocytes in MEM. This suspension was incubated for 30 min at 37°, washed twice in MEM, and 2 ml guinea-pig complement (1:10) was added to the cell pellet. After mixing and a further 30 min incubation in 37° the cells were washed twice and cotton filtered to remove dead cells (see above). The control cell suspension was identically treated except that normal mouse serum was used instead of anti-θ serum.

TABLE 1. Morphological composition of spleen cell suspensions and their survival values in tissue culture

	Normal spleen	'B' spleen	Athymic 'nude' spleen
1. Morphology (without filtration*)			
(a) Lymphocyte-like cells (%)	80	60	45–50
'B' type†	56	55	45
'T' type†	24	4–6	1–2
(b) Other cells (%)			
Large mononuclear cells	3–4	7–8	6–12
Granulocytes	10	10–20	35–40
Immature myelo, erythropoetic and other elements	5	15–25	4–5
2. Lymphocyte survival‡ in culture (%)			
0 hr	100	100	
24 hr	77(90§)	61	n.t.
48 hr	45	35	
60 hr	30(50§)	26	

* After cotton wool filtration (see Materials and Methods) all suspensions contained 90–95% lymphocytes.

† Calculated from the % theta (θ) positive and MBLA positive cells.

‡ Tube cultures, Eagle's medium (expressed as a proportion of viable cell number at 0 hr, at the start of cultures the suspensions contained 90% viable cells).

§ Flat bottomed culture vessels. RPMI-1640 medium (see Materials and Methods). Both 3 and 4 assayed by Trypan blue dye exclusion.

n.t. = not tested.

RESULTS

Characterization of spleen cell suspensions

The three types of spleen cell suspensions used differed in their content of various cell types (Table 1). The approximate proportions of 'T' and 'B' type lymphocytes was calculated from the percentage cytotoxicity induced by anti-θ (i.e. anti-T cell) and anti-MBLA (i.e. anti-B cell) sera in the presence of guinea-pig complement. Although the sum of the proportions of cells killed by these two sera was always approximately equal to the *total* proportion of lymphoid cells it is likely that a sizable proportion of 'lymphocyte-like' cells in B spleens are in fact precursors of haemopoetic cells.

As shown in Table 1 B spleens and athymic ('nude') spleens had a greater proportion of non-lymphoid cells than control normal spleens.

Survival values for the different cell suspensions in culture are also given in Table 1. The survival of B spleen lymphocytes over a 60 hr period was only marginally inferior to that of cells from normal spleens (cf. Doenhoff *et al.*, 1970). Cell viability in flat bottomed vial cultures (with RPMI medium) was consistently better than in tube cultures (with

Eagle's medium) (cf. Adler *et al.*, 1970). No attempt was made to monitor selective survival rates of T and B lymphocytes in the normal spleen cell cultures.

Responses of T and B lymphocytes to phytohaemagglutinin (PHA) and pokeweed mitogen (PWM)

1. *'B' spleen cells compared with normal control spleen cells.* The response of different spleen cell suspensions to varying concentrations of p-PHA and PWM was studied using the tube culture technique (see Materials and Methods). Both RNA synthesis ([^{14}C]uridine uptake) and DNA synthesis ([^{3}H]thymidine uptake) were quantitated. The results are

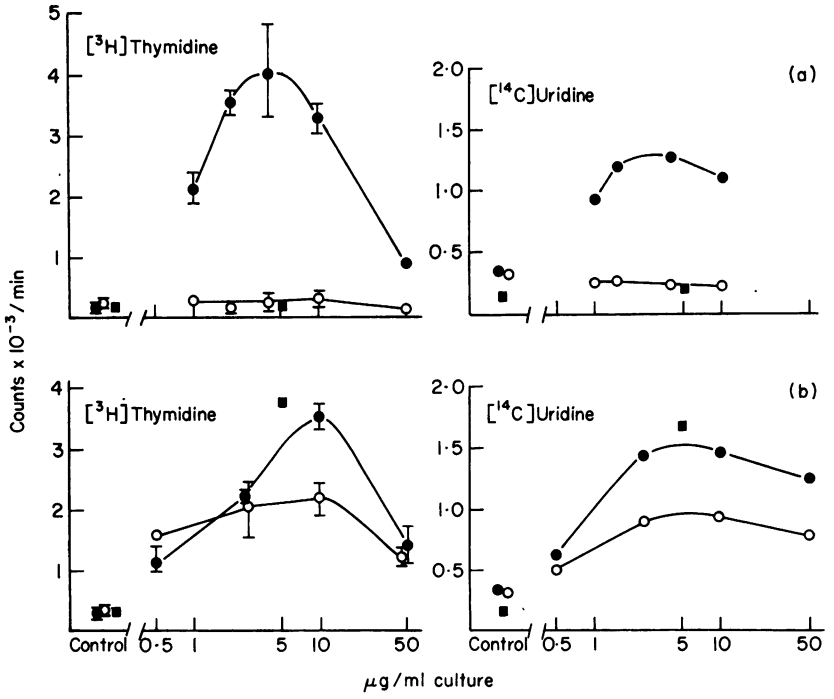

FIG. 1. Activation of lymphocytes by phytomitogens. (a) purified phytohaemagglutinin (p-PHA), (b) pokeweed mitogen (PWM). ●, normal spleen cells; ○, 'B' spleen cells; ■, athymic ('nude') spleen cells. Vertical bars represent 1 standard error of mean. Mean values of two to five cultures given.

shown in Fig. 1. PHA stimulated both RNA and DNA synthesis in the normal spleen cell cultures but induced no significant response in B spleen cell cultures. In contrast, PWM activated both normal and B cultures, the former giving the greater response to the optimal concentration of mitogen*. High concentrations of mitogens induced sub-optimal responses. Viability studies on cultures treated with high mitogen concentrations suggested that reduced responsiveness was not attributable to overt toxicity (PHA-W, in contrast to p-PHA, was however toxic to cells at these concentrations). In further experiments PHA concentrations

* It should, however, be noted that absolute comparison of responsiveness is not valid since the proportion of lymphocytes and contaminating granulocytes in the two groups was unequal.

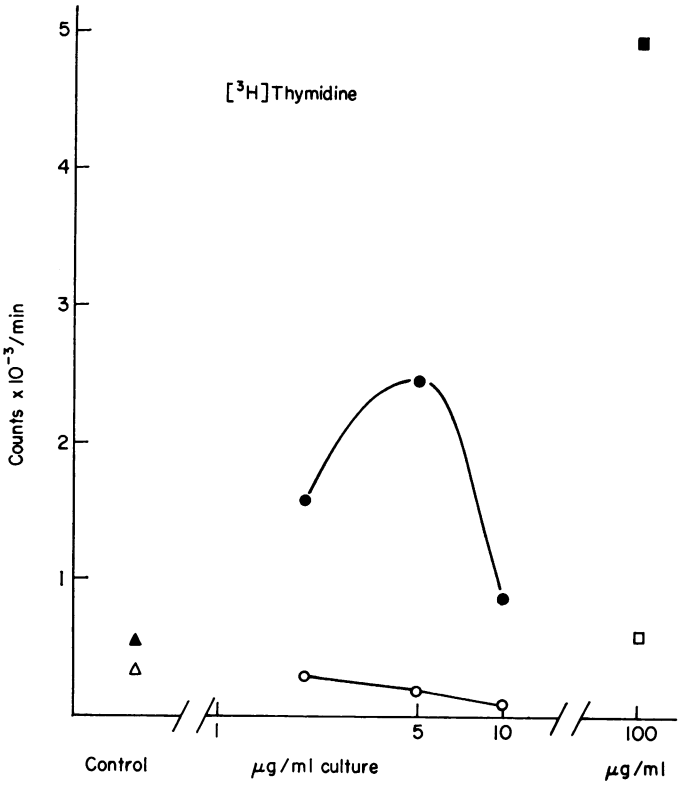

FIG. 2. Early PHA-induced thymidine uptake by lymphocytes (24–28 hr). Controls: ▲, normal spleen cells; △, 'B' spleen cells. p-PHA: ●, normal spleen cells; ○, 'B' spleen cells. PHA-W ■, normal spleen cells; □, 'B' spleen cells (the concentration of PHA-W used was optimal for thymidine uptake: 100 μg/ml, the dose/response curve for PHA-W was similar to that previously reported by Tursi *et al.*, 1969).

TABLE 2. Early response of spleen cell cultures to phytohaemagglutinin

Mitogen	Response, isotope uptake c/min*			
	Normal spleen cells†		'B' spleen cells†	
	[3H]leucine	[14C]uridine	[3H]leucine	[14C]uridine
Control	926± 61	604±48	772±49	214±17
PHA-W‡	1950±105	2159±67	898±26	334±51

* Mean±standard error of mean. Isotope added at 0 hr, and cultures harvested after 24 hr.

† Spleen cells unfiltered. Tube cultures.

‡ At 100 μg/ml (previously determined optimal concentration for [3H]thymidine uptake, 36–60 hr).

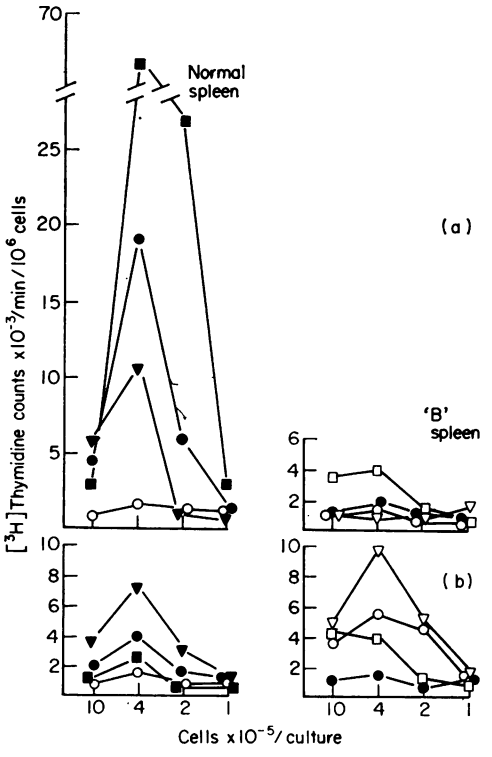

FIG. 3. Effect of cell concentration on response to phytomitogens. (a) phytohaemagglutinin, normal spleen: ■, 100 μg/ml PHA-W; ●, 20 μg/ml p-PHA; ▼, 4 μg/ml p-PHA; ○, controls, 'B' spleen; □, 100 μg/ml PHA-W; ○, 20 μg/ml p-PHA; ▽, 4 μg/ml p-PHA; ●, controls. (b) pokeweed mitogen, normal spleen: ■, 50 μg/ml PWM; ●, 10 μg/ml PWM; ▼, 2·5 μg/ml PWM; ○, controls. 'B' spleen: □, 50 μg/ml PWM; ○, 10 μg/ml PWM; ▽, 2·5 μg/ml PWM; ●, controls.

as low as 0·005 μg p-PHA per ml also failed to activate B cells which are therefore, under the described conditions, resistant to activation by a 10,000-fold range of mitogen concentrations. We next investigated whether varying any of the culture conditions would permit a B cell response to PHA to be expressed. The first point we considered was whether an 'early' (0–48 hr) response of B cells had been missed owing to the design of the experiment. To exclude this possibility we studied early uridine, thymidine and leucine (into proteins) uptake in PHA treated normal and B spleen cultures. The results are given in Table 2 and Fig. 2. These show that during the 48 hr of culture with PHA only very marginal net RNA, DNA or protein synthesis occurred in B spleen cultures in contrast to normal spleen cell cultures which showed uptake values considerably above background.

Although normal spleen and B spleen cultures had similar viability after 60 hr incubation it is theoretically possible that T and B cells have different requirements for optimal *responsiveness in vitro* to proliferative stimuli. As an empirical approach to this problem we have studied the effect of varying cell density and culture media. The effect of different lympho-

TABLE 3. The effect of filtration on incorporation of [^{3}H]thymidine by normal and 'B' spleen cell cultures

Mitogen†	Expt	Response, isotope uptake c/min*			
		Normal spleen cells		'B' spleen cells	
		non-filtered	filtered	non-filtered	filtered
Control	1	1630	766	1820	480
	2	1000	510	1605	382
	3	1790	520	1230	700
PHA-W	1	35200	34300	2860	530
	2	19800	24100	3300	670
	3	35100	39700	5780	1180
PWM	1	24200	21080	4830	5370
	2	12600	10200	6630	6010
	3	28220	16800	11900	6900

* [^{3}H]thymidine added between 36–60 hr of culture. Cultures set up in RPMI-1640/Flow foetal calf serum media in 'Sterilin' flat bottom vials (see Materials and Methods).

† Added at previously determined optimal concentrations (PHA-W: 100 μg/ml; PWM: 5 μg/ml).

cyte concentrations* on PHA and PWM induced thymidine uptake (36–60 hr) is shown in Fig. 3. In this microplate experiment (see Materials and Methods) responsiveness of normal spleen cells to PHA was critically dependent upon cell density as previously reported by Moorhead *et al.* (1967) 4×10^5 cells per 0·2 ml culture fluid appeared to be optimal for both mitogens. The cruder PHA-W preparation induced a considerably better response than p-PHA. The former contains erythroagglutinating activity and this may well have been in part responsible for its greater potency since Yachnin *et al.* (1971) have shown that coating red cells with PHA enhances mitogenicity of the latter.

High concentrations of B spleen cells gave a very small response (approximately 6% of normal spleen) to PHA-W but no response to p-PHA.

Responses to PWM were also dependent upon cell concentration. Normal and B spleen cultures gave essentially similar responses over the varying cell densities and mitogen concentrations. Although only a ten-fold range of cell densities has been studied these results imply that this factor is unlikely to be limiting the PHA responsiveness of B cells.

Earlier experiments had shown that cell survival and background thymidine uptake values were greater when cultures were set up in flat bottomed vials with RPMI-medium as compared to the standard tube culture method with Eagle's medium†. The response of normal and B spleen cells to PHA-W and PWM cultured under these improved conditions is shown in Table 3. In addition the effect of cotton filtration has been studied. It can be

* The relevant parameter being varied is probably cell density per unit area of the culture vessel bottom (cf. Moorhead, Connolly & McFarland, 1967; Watkins & Moorhead, 1969).

† Note that these two media were supplemented with different sources of foetal calf serum (see Materials and Methods).

TABLE 4. Response of normal and athymic ('nude') spleen to phytomitogens *in vitro*

Mitogen†	Response, isotope uptake c/min*			
	Normal spleen cells		'B' spleen cells	
	non-filtered	filtered	non-filtered	filtered
Control	1730	800	540	230
p-PHA	4230	6400	230	280
PHA-W	7600	n.t.	180	n.t.
PWM	15300	10130	1900	7890

* Each value is the mean of duplicate tube cultures (variation between duplicates was <20%). [^{3}H]thymidine, 36–60 hr of culture.

† Added at previously determined optimal proportions (p-PHA: 5 μg/ml; PHA-W: 100 μg/ml; PWM: 5 μg/ml).

n.t. = not tested.

seen that non-filtered B spleen cultures did in fact give a small response to PHA which was approximately twice background. In one case the PHA induced response of B spleen was more marked. Cotton filtration (see Materials and Methods) of the cells prior to culture had three interesting effects: (a) background uptake was considerably reduced, (b) the PHA induced response of B spleen, but not of normal spleen cultures, was lost and (c) PWM induced responsiveness of B spleen cultures was unimpaired. This experiment suggests, therefore, that under certain culture conditions a small PHA induced response of B spleen cells can be shown which is apparently dependent to a large extent upon the presence of 'adhesive' cells. There are several possible interpretations of this observation (see Discussion). It is clear however that under the different conditions of culture investigated the response of B spleen cells to PHA was always less than 15% of the normal spleen cultures, despite the fact that both responded well to PWM.

2. *Responses of athymic 'nude' spleen cells compared with normal spleen cells.* Spleens of congenitally athymic 'nude' mice have been shown to be deficient in T cells (Raff & Wortis, 1970). The majority of lymphoid cells in 'nude' spleens have the surface characteristics of B cells, i.e. θ-negative, MBLA positive (see Table 1 and Raff & Wortis, 1970). Furthermore, Wortis (1971) has shown that, in contrast to normal (or heterozygous) litter mate controls, 'nude' mice do not produce a lymph node proliferative response following the injection of PHA.

The responsiveness of 'nude' spleen cells to p-PHA and PWM *in vitro* is shown in Table 4. In contrast to normal spleen cell cultures, spleen cells from 'nude' mice were not activated by PHA. They did however respond to PWM. Reactivity was considerably enhanced by cotton filtration prior to culture implying that granulocytes or other adhesive cells present in considerable numbers in spleens of these athymic mice (see Table 1) may depress the proliferative response of B-cells.

3. *Affect of anti-θ (theta) serum on the responsiveness of normal spleen cell suspensions to PHA and PWM.* Anti-θ serum selectively lyses T cells in the presence of guinea-pig complement (Raff, 1969). We therefore anticipated that the treatment of normal spleen cells

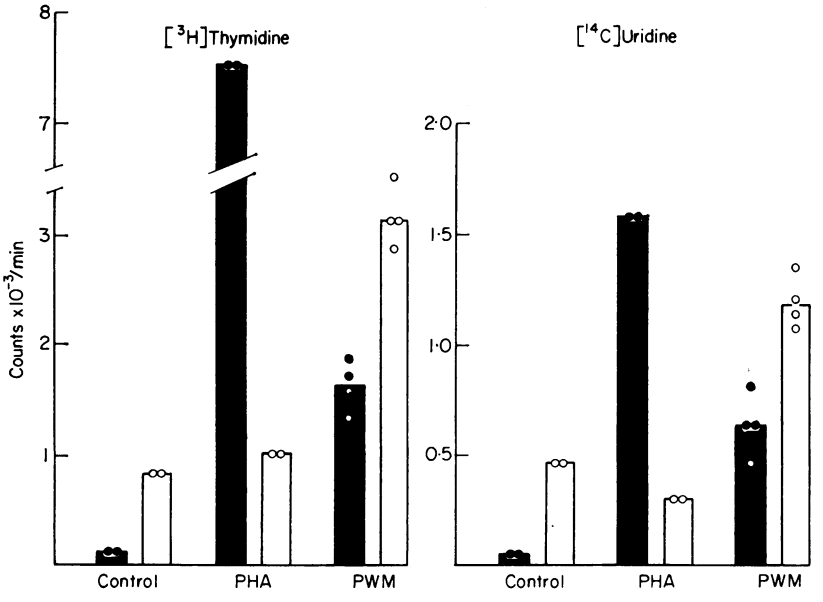

FIG. 4. Effect of anti-θ serum on the response of spleen cells to phytomitogens. ■, cells pretreated with control (AKR) serum plus complement, □, cells pretreated with anti-θ serum plus complement. Each point represents an individual culture.

with these reagents prior to culture would abolish the PHA response. As shown in Fig. 4 the PHA response was in fact reduced by 90% by anti-θ serum. In marked contrast, the PWM response was enhanced by pretreatment with anti-θ serum implying that T cells may make little contribution to the PWM response of normal spleen cell cultures. Thymidine and uridine uptake were similarly affected. It is also interesting to note that anti-θ serum plus complement itself induced a proliferative response (approximately five times background). We suppose that this effect is due to activation of that small proportion of T cells which escaped the lytic effect of anti-θ serum. It will be of interest in this respect to study the activation of lymphocytes by anti-θ serum under non-lytic conditions.

DISCUSSION

These results demonstrate that whereas phytohaemagglutinin (PHA) selectively activates thymus-derived (T) cells, pokeweed mitogen (PWM) activates 'bursa-equivalent'-derived (B) cells. T cells may also respond to PWM although the evidence for this in the above experiments was equivocal*. The selective nature of activation induced by the two mitogens was manifested by RNA, DNA and protein synthesis, and was evident over several days in tissue culture with a wide range of mitogen concentrations. The results were essentially the same with all three sources of B cell suspensions: (a) anti-θ serum treated 'normal' spleens, (b) congenitally athymic mouse spleens and (c) spleens from adult thymectomized mice, lethally irradiated and reconstituted with anti-θ serum treated syngeneic bone marrow suspensions (B-spleens).

* Recent experiments (Janossy & Greaves, unpublished) with thymocytes from cortisone-treated mice have shown that PWM does in fact stimulate T cells.

TABLE 5. A summary of direct and circumstantial evidence for selective activation of 'T' and 'B' lymphocytes by phytohaemagglutinin and pokeweed mitogen

Test system	Species	T and/or B cells implicated		References
		PHA	PWM	
1. Cell markers				
i. chromosomal				
T6T6	mouse	T		Doenhoff *et al.* (1970)
sex (y)	rat	T		Johnson & Wilson (1970)
ii. Theta (θ) surface antigen—susceptibility to anti-θ serum				
(a) Pretreatment of cells prior to culture	mouse	T	B	This study*
(b) Cytotoxicity of stimulated cells	mouse	T		Owen, J.J.T. (personal communication)
2. Experimental immunological deprivation				
i. neonatal thymectomy	mouse	T		Martial-Lasforgues *et al.* (1966), Dukor & Dietrich (1967), Rodey & Good (1970), Takiguchi *et al.* (1971)
	rat	T		Rieke (1966), Schwarz (1968), Meuwissen *et al.* (1969b)
	chicken	T		Greaves, Roitt & Rose (1968), Meuwissen *et al.* (1969a)
ii. Adult thymectomy plus anti-lymphocyte globulin	mouse	T		Tursi *et al.* (1969)
iii. Adult thymectomy plus 'B' cell reconstitution	mouse	T	B	This study
iv. Bursectomy	chicken	T		Greaves *et al.* (1968), Alm & Peterson (1969), Meuwissen *et al.* (1969a)
3. Congenital or 'naturally' acquired immunological defiiency				
i. cell-mediated e.g. thymic aplasia	mouse	T	B	This study
	man	T		Oppenheim (1968), Gotoff (1968), Douglas, Kamin & Fudenberg (1969), Bach *et al.* (1969)
ii. humoral e.g. agammaglobulinaemia (Bruton)	man	T	B	
4. Tissue distribution of responsive cells				
i. thymus cf. bursa	chicken	T		Weber (1967)
ii. thymus cf. peripheral tissue/circulating cells	rat	T		Colley, Shih Wu & Waksman (1970)
	man	T		Claman (1966), Pegrum, Ready & Thompson (1968)
iii. Gut associated ('B' type?) tissue cf. other lymphoid tissue	mouse	T		Rodey & Good (1970)
5. Characteristics of the response				
i. Polyribosomal vs. polydisperse RNA synthesis	man	T	B	Chessin *et al.* (1966)
ii. Ultrastructure—rough endoplasmic reticulum	man	T	B	Chessin *et al.* (1966), Barker, Lutzer & Farnes (1969)
iii. Immunoglobulin synthesis	mouse	T	B	Parkhouse, Janossy, & Greaves (to be published)
	man	T	B	Greaves & Roitt (1968)

* In a recent study, Blomgren & Svedmyr (1971) have also described the inhibitory effect of anti-θ serum in the PHA response.

Unfiltered B-spleen suspensions were heterogeneous and contained a high number of myelo- and haemopoetic elements and other cells. However morphological studies on cultured cells (Janossy & Greaves, unpublished) and an analysis of immunoglobulin synthesis (Parkhouse, Janossy & Greaves, unpublished) suggest that most, if not all, of the PWM induced isotope uptake is due to B lymphocyte activation and concomitant differentiation along the plasma cell line (cf. Douglas *et al.*, 1967). In addition cotton wool filtered suspensions containing 90–99% lymphocytes responded to PWM. Despite the presence of a small proportion of residual T (θ positive) cells in these suspensions (1–6%), isotope uptake was not enhanced by PHA under the standard tube culture conditions.

The observed specificity of PHA for T cells is in accord with other direct and circumstantial evidence which has been tabulated in Table 5. In the present experiments B cells were cultured in the absence of T cells and the results do not rule out the possibility that B cells will respond either directly or indirectly when mixed with T cells. Other studies, however, using chromosomal and antigenic markers (Table 5) suggest that B cells make, at the most, only a very limited mitotic response to PHA even in the presence of activated T cells.

Previous evidence (Table 5) for responsiveness of B cells to PWM has been equivocal, although Douglas *et al.* (1969) described a depressed response of lymphocytes from agammaglobulinaemic individuals which nevertheless reacted to PHA. The experiments described above showed that purified B cell cultures do respond well to PWM. The lack of responsiveness of B cells to PHA cannot therefore be attributed to poor viability or lack of proliferative capacity *in vitro*. We feel that these observations provide an explanation for some of the observed discrepancies in the characteristics of the response of cells to PHA and PWM (Table 5). In particular it provides a basis for the observation that PWM stimulated cells synthesize polyribosomal RNA and develop rough endoplasmic reticulum (Chessin *et al.*, 1966; Douglas *et al.*, 1967)—features characteristic of the plasma cell (B) line. Earlier studies with human lymphocytes suggested that PWM but not PHA activated immunoglobulin synthesis in a small proportion of the cultured cells (Greaves & Roitt, 1968). We are currently investigating immunoglobulin synthesis and secretion in mouse lymphocyte cultures in the presence of PHA and PWM and preliminary results confirm the above mentioned study in that PWM cultures synthesize and secrete considerably more immunoglobulin (principally IgM) than controls whereas little or no enhanced synthesis was observed with PHA stimulated cultures.

It seems reasonable to conclude that PWM activated B cells, or at least a substantial proportion of them, undergo a similar pattern of differentiation as they would normally do only when stimulated by ligands functioning as specific antigens. Similarly the pattern of T cell responsiveness to PHA is similar, if not identical, to that observed when the same population is actively engaged in cellular hypersensitivity reactions *in vivo* or *in vitro*, induced by antigen.

While this study has shown that selective effects of phytomitogens exist, we do not wish to conclude that specificity of the stimulants is necessarily absolute. Although we have investigated such parameters as cell density, culture media, culture tubes/containers etc. in addition to time course studies and the effects of a wide range of mitogen concentrations (10^4), it is still theoretically possible that conditions might be found under which both populations would respond to either phytomitogen. In one of the three culture methods we investigated a very small but significant response to PHA was observed with B spleen cell suspensions. This method involved a foetal calf serum with a high stimulating capacity as

manifested by the high background control values for thymidine uptake. Cell survival and responsiveness was also facilitated by the use of a flat bottomed culture vessel and RPMI-1460 medium. Background values and the PHA induced increase of thymidine uptake were both considerably reduced by cotton wool filtration of the suspensions prior to culture. We suspect that the high background and slight PHA response of unfiltered cells is attributable to a macrophage dependent immune response to foetal calf serum proteins and perhaps also to PHA itself. Despite the lack of PHA enhanced isotope uptake in cotton wool filtered B spleen cell suspensions, we have observed some cellular transformations involving 7–10% of the cells after 60 hr culture. The morphological appearance of these cells suggests that they are plasmablasts and they are therefore probably indicative of a small abortive PHA response of a minor population of B cells rather than a reaction of the small residual T cell population.

The biochemical basis of the selectivity of phytomitogens is unresolved. In this respect it is of interest to quantitate binding sites for PHA and PWM on T and B cells since these could provide a basis for the observed specificity. However our preliminary results suggest that the unresponsiveness of B cells to PHA cannot be ascribed to a lack of PHA binding sites. Another possibility is that the strong agglutination of B cells may have inhibited their response to PHA; PWM is a much weaker leucoagglutinin.

Caution is necessary in extrapolating these results to other species, although we suspect the observed relationship of PHA/T cells and PWM/B cells to be true for lymphocytes for human beings (cf. Douglas *et al.*, 1969). The rat in particular may be different in that the PWM response is almost completely ablated by thymectomy (Schwarz, 1968; Meuwissen *et al.*, 1969).

We suggest that studies on selective activation of T and B cells by phytomitogens may provide clues as to the nature of antigen induced activation and differentiation of these cell lines *in vivo*. In addition responses to PHA, PWM and other stimulants may serve as useful indicators of the proliferative potential of T and B cells in diseases or clinical procedures associated with immunological deficiency.

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